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Relationships between oxidation and [mu]-calpain autolysis, degradation of Ca[superscript]2+-regulating proteins, and pork quality

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Relationships between oxidation and μ-calpain autolysis, degradation of Ca^{2+}-regulating proteins, and pork quality

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General Introduction

The production of meat from farm to fork is one of the largest industries in the United States. In terms of total consumption of meat, poultry, and fish, Americans have increased their per capita consumption steadily from 175.7 pounds in 1970 to 230.8 pounds in 2001 (Pork Facts, 2002/2003). Although people are eating more total meat, the amount of pork being consumed per capita in the United States has stayed relatively stable from 1970 to 2001 (Pork Facts, 2002/2003). In order to increase the amount of pork being consumed, much work has been done to improve the quality of pork being produced. Factors such as feeding strategies, stress, genetics, and how the pig is handled/transported can all influence pork quality before the pig arrives to the harvest facility (Berg 1998). Factors such as lariage times, feed restriction, stunning methods, and carcass chilling (among other factors) can ultimately affect pork quality once the pig reaches the harvest facility and is harvested (Berg 1998). Less desirable pork classifications such as pale, soft, and exudative (PSE) and dark, firm, and dry (DFD) pork can be a direct result of negative environmental and managerial factors to which the pig is exposed prior to, during, and after harvest.

One of the factors contributing the development of PSE or DFD pork is that of the rate and extent of postmortem glycolysis. Glycolysis is the anaerobic metabolism of glucose that results in the production of lactic acid, which contributes to the decrease in pH of the pork carcass postmortem. DFD pork characteristically has a very minimal pH decline due to relatively low glycogen stores available to be used in glycolysis. PSE pork is a result of increased metabolism in the muscle, which results in a very rapid pH decline and the ultimate pH being reached while carcass temperature is still high. This environment found in PSE

pork can have negative effects on color, texture, and drip loss. Hams produced from PSE muscles may have 5%-10% lower processing yields, due to its lack of ability to bind water (Buege 1998).

Biochemical factors such as calcium regulation by the ryanodine receptor and sarcoplasmic reticulum Ca^{2+}-ATPase pump (SERCA) within the cell in the time postmortem can affect the rate of the pH decline. Very severe PSE can result from a mutation in the ryanodine receptor, the protein responsible for Ca^{2+} release into the sarcoplasm. The troponin complex utilizes this sarcoplasmic Ca^{2+} to initiate muscle contraction (Goll et al., 1984). A large influx of Ca^{2+} from the ryanodine receptor into the sarcoplasm results in a higher rate of muscle metabolism and a more rapid pH decline. The SERCA pump is primarily responsible for the removal of Ca^{2+} from the sarcoplasm and returning it to the sarcoplasmic reticulum. A loss of activity of the SERCA pump in removing Ca^{2+} used for muscle contraction can also aid in creating the environment for poor quality pork.

Proteolysis of specific proteins by the ubiquitous enzyme μ-calpain has also been shown to be related to both tenderness and water-holding capacity. μ-Calpain undergoes autolysis upon its activation, resulting in μ-calpain molecules degrading other μ-calpain molecules (Zimmerman and Schlapfer, 1991; Cottin et al., 2001). The intermediate filament protein desmin is responsible for muscle structure and integrity (Robson, 1995). The increased degradation of desmin by μ-calpain has been linked to increased water-holding capacity (Rowe et al., 2001) and increased tenderness (Koohmaraie et al., 1984; Ho et al., 1996; Huff-Lonergan et al., 1996; Rowe et al., 2001; Melody et al., 2003). The Ca^{2+} regulating proteins ryanodine receptor (Gilchrist et al., 1992; Iino et al., 1992; Shoshan-Barmatz et al., 1994; Brandt et al., 1992) and SERCA pump (Zhao et al., 1997; Seidler et al.,

1998) are also susceptible to degradation by μ-calpain. Factors such as pH (Rowe et al., 2001; Melody et al., 2003), ionic strength (Geesink and Koohmaraie, 2000), and the amount of its natural inhibitor calpastatin present (Geesink and Koohmaraie, 1999) in the muscle can influence the activity and subsequent degradation of proteins by μ-calpain. An objective of this study was to study how μ-calpain autolysis may relate to the degradation of SERCA, ryanodine receptor, and desmin.

Oxidative environments in the muscle can be detrimental to the function of the ryanodine receptor, SERCA pump, and μ-calpain. Increased levels of oxidation can decrease the activity of the SERCA pump, resulting in less Ca^{2+} being returned to the sarcoplasmic reticulum (Klebl et al., 1998; Xu et al., 1997; Grover et al., 2003). A loss of control of the ryanodine receptor can also be observed when this Ca^{2+} channel is oxidized, causing a large release of Ca^{2+} into the sarcoplasm (Donoso et al., 2000; Feng et al., 1998; Heunks et al., 2001; Zable et al., 1997; Oba et al., 2002). Oxidation has also been shown to be detrimental to μ-calpain activity and the rate of autolysis, but not necessarily the extent of autolysis (Guttmann et al., 1997; Guttmann and Johnson 1998; Rowe et al., 2002). Another objective of this project was to determine the relationships between oxidation and the autolysis of μ-calpain and degradation of SERCA-ryanodine receptor, and desmin.

Supplementation of an antioxidant into the swine diet prior to harvest may be able to aid in preventing some of the factors present that could detrimentally affect pork quality. The thiol-based antioxidant α-lipoic acid has been shown to be a potent free radical scavenger in the cell (Packer et al., 1995). α-Lipoic acid has also been observed to have vitamin E recycling abilities in the cell (Kagan et al., 1992). Supplementation of α-lipoic acid may also affect glycogen storage in the muscle as α-lipoic acid displays insulin-like

properties in stimulating glucose storage as glycogen (Jacob et al., 1995; Estrada et al., 1996), which may aid in the availability of glycogen to be used in anaerobic metabolism. In a previous study, α-lipoic acid has been shown to positively affect pork quality attributes such as pH, L* values, and drip loss (Berg et al., 2001). Based on these properties of α-lipoic acid, an objective of this study was to determine the effect of α-lipoic acid supplementation on influencing fresh pork quality.

Thesis Organization

This thesis is in an alternative style format consisting of a general introduction, a review of the literature, paper prepared for publication, and a concluding summary. The paper represents the work done by the first author to fulfill the requirements for the degree of Master of Science. The paper was prepared according to the Meat Science Style and Form guide. This paper consists of an abstract, introduction, materials and methods, results and discussion, and literature cited sections.

GENERAL REVIEW OF LITERATURE

Glycogen Storage in Skeletal Muscle

The storage form of glucose within the muscle is glycogen. Glycogen is made up of α1-4 and α1-6 linkages of glucose molecules. The synthesis of glycogen involves a protein primer, glycogenin, which acts as a hexosyltransferase (Alonso et al., 1995). Glycogen synthesis begins as tyrosine-194 on the glycogenin residue becomes autocatalytically glucosylated by the addition of UDP-glucose into the system (Lomako et al., 1993). On average, seven other glucose residues are added to tyrosine-194 in the same manner. These eight total glucose residues become the primer for the synthesis of proglycogen. Proglycogen is a stable intermediate for both the synthesis and breakdown of glycogen (Alonso et al., 1995). This stable intermediate is formed by the addition of proglycogen synthase and branching enzyme to the fully glucosylated glycogenin molecule. Proglycogen is then converted to macroglycogen, the storage form of glucose, by using macroglycogen synthase and branching enzyme (Lomako et al., 1993). This reaction is reversible, allowing for the breakdown of macroglycogen into proglycogen by use of phosphorylase and debranching enzyme (Lomako et al., 1993). In terms of metabolical activity, the larger macroglycogen (10^7 D) is inactive while proglycogen (400,000 D) is an active intermediate (Alonso et al., 1995). In order for glycogen to be used in metabolism, macroglycogen must first be converted into proglycogen.

A schematic of this process is displayed below as adapted by Lomako and others in 1993 (Figure 1).

The implication of this glycogen storage mechanism is that glycogen storage may slow down the rate of glycolysis by storing more glucose as the inactive macroglycogen as opposed to the metabolically active proglycogen. In a study done by Huang and others (1997), rats were exposed to minimal levels of stress that resulted in proglycogen being exclusively depleted. Proglycogen was also the only molecule that underwent synthesis and degradation when the rate of glycogen turnover was low (Huang et al., 1997). As the stress levels increased, macroglycogen was recruited in glycogen turnover. This study also observed that injecting high levels (greater than 10 mU/min^{-1}) of the hormone insulin promoted the storage of glucose in the form of macroglycogen as opposed to proglycogen.

Glycogen can be utilized for glycolysis once it has been converted from the storage form of macroglycogen to the metabolically active form of proglycogen. The mechanisms behind postmortem glycolysis are very complex but have been shown to affect many factors associated with pork quality. A discussion of the events that occur during postmortem glycolysis will now be discussed.

Anaerobic Metabolism of Glucose

Glycolysis is the pathway for providing energy from the stepwise degradation of glucose (Garrett and Grisham, 1999). It is a primarily anaerobic form of metabolism and produces short-term energy when oxygen is limiting to the organism. Glycolysis consists of two phases (Garrett and Grisham, 1999). The first phase is a series of five reactions in which glucose is broken down to two molecules of glyceraldehyde-3-phosphate. This phase consumes 2 molecules of ATP. The second phase converts the two molecules of

glyceraldehyde-3-phosphate into two molecules of pyruvate. The second phase results in a net gain of four ATP, thus glycolysis results in a net gain of two ATP.

In animal tissues that are under anaerobic conditions, the two molecules of pyruvate that are produced by glycolysis are reduced to lactate. This occurs when the pyruvate can no longer be oxidized by the TCA cycle due to a lack of oxygen. The pyruvate is then reduced by lactate dehydrogenase to produce the acidic lactate.

This pathway accounts for the reduction in the pH of post-mortem muscle as it enters rigor. As muscle is converted to meat, exsanguination results in a lack of oxygen to the muscle tissue due to a loss in function of the circulatory system. As oxygen is depleted, a shift from aerobic metabolism to anaerobic metabolism occurs as the body is still trying to produce ATP and maintain function. This results in a less efficient production of ATP. As a direct result, lactic acid begins to accumulate due to the circulatory system not being able to remove the lactic acid from the muscle where it would normally be taken to the liver to be converted back to glucose (Goll et al., 1984).

The rate of pH decline is related to the temperature of the muscle. A higher muscle temperature will result in a faster rate of pH decline (Pearson, 1987). A lower muscle temperature will result in the opposite affect, resulting in a slower rate of pH decline. Once the ATP is completely consumed, and no more is being produced, permanent cross-bridges are formed between myosin and actin to create actomyosin. This condition is termed rigor mortis.

Permanent cross-bridges between actin and myosin that constitute rigor mortis are a result of biochemical mechanisms that occur during muscle contraction and muscle relaxation. The extent of muscle contraction can affect many aspects of pork quality and can

be related to postmortem metabolism of the muscle. A strong muscle contraction can result in a higher rate of anaerobic metabolism and a larger amount of lactic acid being produced in during early timepoints postmortem, causing a more rapid pH decline.

Contraction of Skeletal Muscle

The events that lead to the contraction of skeletal muscle involve a nerve impulse that is propagated along the motorneuron to the terminus of the axon at the neuromuscular junction (Goll et al., 1984). This nerve impulse causes the release of acetylcholine from presynaptic vesicles in the axon terminus, which ultimately results in the depolarization of the motor end-plate region (Goll et al., 1984). This depolarization is extensive enough to depolarize the adjacent sarcolemmal membrane, which leads to a depolarization of the transverse tubules (T- tubules) found in the muscle cell membrane (Goll et al., 1984). The T-tubule is a distinct membrane structure that connects to the terminal cisternae of the sarcoplasmic reticulum (SR) and its function is to conduct the neural signal to the interior of the muscle cell for contraction (Goll et al., 1984). The mechanism by which this action potential is propagated along the muscle plasma membrane is called excitation-contraction coupling (Goll et al., 1984). Electron micrographs show that the T- tubules are located at approximately 5 nm from the lateral cisternae in the SR, which contains up to 90% of the Ca^{2+} found in the resting muscle cell (Goll et al., 1984). When the T- tubule becomes depolarized, the result is a release of this Ca^{2+} from the lateral cisternae into the sarcoplasm.

Dihydropyridine Receptor and Ryanodine Receptor

This process of excitation-contraction coupling has been researched at the membrane level. The passage of Ca^{2+} involves the dihydropyridine (DHP) receptor found in the T-tubule and the sarcoplasmic reticulum release channel also known as the ryanodine receptor. The DHP receptor is a tetrad receptor that is similar to the voltage dependant sodium channels in that each of the four repeated segments has five hydrophobic segments and one positively charged segment (Tanabe et al., 1987) in its alpha one subunit. The positive charge in this segment may act as a voltage sensor for the DHP receptor.

The 565 kDa ryanodine receptor (RYR) protein is classified as a tetramer that contains a foot structure that bridges the gap between the sarcoplasmic reticulum and T. tubule membranes. The secondary structure of the protein suggests that the C-terminal end inserts into the sarcoplasmic reticulum membrane while the rest of the protein is cytoplasmic and lies between the T. tubule and sarcoplasmic reticulum (Best et al., 1991). This cytoplasmic portion is known as the foot structure. This receptor protein acts as a calcium channel that is activated by submicromollar concentrations of Ca^{2+} and millimolar ATP. A protein-protein interaction between the DHP receptor and the RYR is believed to exist, suggesting that the signal for Ca^{2+} release from the RYR is passed on by the DHP receptor.

In mammals, there are three different isoforms of the ryanodine receptor: RYR1, RYR2, and RYR3 (Fill and Copello, 2002). The predominant isoform found in skeletal muscle is the RYR1 isoform. RYR2 is abundantly found in cardiac muscle. RYR3 is found in striated muscles but at relatively low levels (Froemming et al., 2000). However, RYR3's physiological role is not yet clear. Mice that were missing RYR1 or RYR2 gene products die

during early embryonic development (Takeshema et al., 1998). In contrast, mice missing RYR3 lead relatively normal lives with normal striated muscle (Takeshema et al., 1998). This may suggest that RYR1 and RYR2 somehow compensate for the missing RYR3 isoform. The primary structures of the three isoforms share approximately 70% identity (Hakamata et al., 1992).

Calmodulin is a small (17 kDa), ubiquitously expressed Ca^{2+} binding protein that appears to act as a Ca^{2+} sensor in eukaryotinc cells (Celio et al., 1996) and has been found to interact with single ryanodine receptors in lipid bilayers (Smith et al., 1986). Calmodulin binds to the ryanodine receptor that is ~10 nm from the entrance to the transmembrane pore of the ryanodine receptor (Wagenknecht et al., 1989). Calmodulin has been shown to activate the ryanodine receptor at submicromolar cytosolic Ca^{2+} concentrations while inhibiting ryanodine receptor functions at higher Ca^{2+} concentrations (Tripathy et al., 1995).

Another protein that may affect ryanodine receptor function may be that of calsequestrin. However, there is disagreement of the exact nature of calsequestrin-ryanodine receptor interaction. It has been suggested that Ca^{2+} concentration (Culligan et al., 2002) and pH dependant (Hidalgo et al., 1996) modulations in calsequestrin modulate ryanodine receptor activity. However, it has also been suggested that calsequestrin may require the presence of triadin in order to regulate ryanodine receptor activity (Zhang et al., 1997).

There is strong evidence suggesting the interaction of the DHP receptor and the ryanodine receptor. Proenza et al. in 2002 researched the interaction of the DHP receptors and ryanodine receptor. It has been hypothesized that a voltage-dependant conformational change in the II-III loop in the alpha one subunit of the DHP receptor allosterically activates the RYR1 isoform of the ryanodine receptor. Proenza and others (2002) used a yeast two-

hybrid system of assays to observe direct binding of the two receptors. They were able to determine an interaction between the s53 region of the II-III loop of the DHP receptor (amino acids 720-765) and the sR16 region (amino acids 1837-2168 on R10) of the ryanodine receptor. This finding supports the idea that there is a protein-protein interaction between the DHP receptor and the ryanodine receptor. This allosteric modification of the ryanodine receptor allows for a release of Ca^{2+} from the lateral cisternae of the sarcoplasmic reticulum to enter the sarcoplasm. As a result of this Ca^{2+} release, free Ca^{2+} concentration in the sarcoplasm rises from 10^{-8} M to 10^{-6} M, thus signaling contraction.

The Troponin Complex

The troponin complex consists of the proteins troponin-I, troponin-T, and troponin-C. In the absence of Ca^{2+}, troponin-T binds tightly to tropomyosin and troponin-C binds loosely to troponin-I. Also, troponin-T and troponin-I are firmly bound to actin and loosely bound to troponin-T (Goll et al., 1984). This arrangement of the troponin complex blocks the myosin-binding site on actin in the absence of Ca^{2+}. However, when Ca^{2+} is added to the complex, troponin-C becomes tightly bound to troponin-I and troponin-T. Troponin-I then loses its affinity for actin while retaining its loose linkage to troponin-T (Goll et al., 1984). This results in the tropomyosin shifting and exposing the binding site on the myosin head, allowing for an interaction of the myosin with actin. The interaction of myosin (thick filament) and actin (thin filament) through cross-bridges and allows development of a contractile force, pulling the actin filaments towards the center of the sarcomere (Aberle et al., 2001).

Roles of ATP in Muscle Contraction

ATP serves several roles in muscle contraction (Goll et al., 1984). One role of ATP is to provide energy for contraction by its hydrolysis to ADP and inorganic phosphate. Its second role is to prevent the actin-myosin interaction and even dissociates the crossbridges that form between actin and myosin. In order for contraction to occur, the ADP and inorganic phosphate on myosin must be released from myosin. This occurs as a result of Ca^{2+} being released into the sarcoplasm and tropomyosin is moved so corssbridges between actin and myosin can occur. Once crossbridges are formed, the ADP and inorganic phosphate are released. After these products are released, a new ATP can bind to the myosin head. This ATP is a powerful dissociator of the actin-myosin crossbridge and the actin is relased from myosin, initiating relaxation and resulting in ATP being hydrolyzed back to ADP and an inorganic phosphate (Goll et al., 1984).

In aerobic organisms, a majority of the production of ATP comes from the aerobic TCA cycle, which produces sufficient amounts of ATP for relaxation of the muscle (Berg et al., 2002). However, in postmortem anaerobic muscle, production of ATP switches from the aerobic TCA cycle to the anaerobic glycolysis cycle (Berg et al., 2002). Glycolysis yields less ATP and eventually the amount of ATP required by the muscle to relax is greater than the amount being produced by glycolysis. This results in permanent crossbridges being formed between actin and myosin, known as rigor mortis.

Removal of Ca^{2+} by the Sarcoplasmic Reticulum Ca^{2+}-ATPase pump

The series of events that make up the contraction process continues until Ca^{2+} is removed from the sarcoplasm. The removal of Ca^{2+} causes troponin-C to no longer bind to Ca^{2+}, troponin-I to interact directly with actin, and tropomyosin to cover the myosin-binding sites on actin (Goll et al., 1984). This removal of Ca^{2+} from the sarcoplasm involves the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump.

The SERCA pump comprises nearly 80% of the sarcoplasmic reticulum membrane (Berg et al., 2002) and will function as long as ATP is present during early post mortem periods. During these early periods postmortem, it is critical to maintain a low level of Ca^{2+} within the sarcoplasm (Mickelson and Louis, 1993). An increased amount of Ca^{2+} in the sarcoplasm may lead to an increased rate of glycolysis and a faster pH decline. The SERCA pump will maintain a Ca^{2+} concentration of 0.1 μM in the sarcoplasm compared with 1.5 mM in the sarcoplasmic reticulum while the muscle is at rest (Berg et al., 2002).

The SERCA pump can be visualized as containing a cytoplasmic headpiece, a stalk, and a transmembrane base, with most of the 110-kDa mass of the protein residing in the sarcoplasm (Mickelson and Louis, 1993). The cytoplasmic headpiece consists of approximately 50% of the molecular weight of the protein and has three domains: N, P, and A. The N domain's function is to bind ATP. The P domain accepts a phosphoryl group on its aspartate-351 residue. The A domain functions as an actuator for the N domain.

The SERCA pump can interconvert between two different conformations called E1 and E2. The mechanism of removing Ca^{2+} from the sarcoplasm begins as two Ca^{2+} ions bind to the E1 state and ATP binds to the N domain. Sarcoplasmic reticulum Ca^{2+}-ATPase then

cleaves the ATP and transfers a phosphate group to the Asp-351 on the P domain. This reaction only occurs in the presence of relatively high Ca^{2+} concentration in the sarcoplasm and once Ca^{2+} is bound to the E1 phase of the SERCA pump. This reaction results in the E1-P conformation. The slow conversion of E1 conformation to E2 conformation causes and inversion of the ion binding sites, so that Ca^{2+} ions can only dissociate into the sarcoplasmic reticulum and not back into the sarcoplasm. This is known as the E2-P conformation. This E2-P phase has a low affinity for Ca^{2+} and the two Ca^{2+} ions are released into the sarcoplasmic reticulum. Upon release of the Ca^{2+}, the phosphoryl group is hydrolyzed and is released from the P domain, resulting in the E2 conformation. When the enzyme is lacking the bound phosphoryl group, it is unstable in its E2 form and will invert back to its E1 phase, thus completing the cycle and allowing for more Ca^{2+} to be removed from the sarcoplasm.

Molecular cloning analysis reveals that SERCA pump can be found in three different isoforms, encoded by a highly conserved family of genes (Loukianov, et al., 1998). In each of the isoforms, the genes encode two alternatively spliced transcripts (Loukianov et al., 1998). In adult tissue, SERCA-1a proteins are exclusively expressed in fast-twitch skeletal muscle (Brandl et al., 1987). The SERCA-2 gene encodes for the SERCA-2a and SERCA-2b isoforms. SERCA-2a is found exclusively in cardiac, slow-twitch skeletal muscle, and smooth muscle (Brandl et al., 1987). SERCA-2b is expressed in most cell types but found in high levels in smooth muscle (Lytton et al., 1989). SERCA-3a and SERCA-3b are expressed in specialized cell types such as platelets, lymphocytes, endothelial cells, and epithelial cells (Wuytack et al., 1995). In adult fast-twitch skeletal muscle sarcoplasmic reticulum, the SERCA-1a isoform is expressed at three to five times higher level than the SERCA-2a isoform in either slow-twitch or cardiac muscle (Wu et al., 1993). This difference in pump

density may partly account for the different Ca^{2+} uptake of fast and slow twitch muscle (Loukianov et al., 1998).

Phospholamban is an intrinsic membrane protein that has been shown to mediate the effects of β-adrenergic agonists on the SERCA-2 isoform. This membrane protein comprises of 52 amino acids and its hydrophobic C-terminal end is believed to be located near the center of the transmembrane portion of SERCA-2 (Hutter et al., 2002). This protein maintains the SERCA in an inhibited state. Phospholamban is dissociated from SERCA by phosphorylation of Ser 16 of phospholamban (Hutter et al., 2002; Kubo et al., 2002). By using 3-D structures of both proteins and applying energy minimization calculations to obtain structural details, phosphorylation may be breaking salt and hydrogen bond linkages that exist between the SERCA and phospholamban (Hutter et al., 2002).

In a similar fashion, SERCA-1 is inhibited by the 31 amino acid transmembrane protein sarcolipin (Tupling et al., 2002). In a study done by Tupling and others (2002), it was found that overexpression of sarcolipin (by IM injection) in rat skeletal muscle reduced Ca^{2+} uptake by 31% when compared with the control rats. It was also observed by using isometric twitch properties, that muscles with overepressed sarcolipin were more susceptible to fatigue and had depressed muscle activity (Tupling et al., 2002).

Effect of Stress on Ca^{2+} Release and Ca^{2+} Uptake

Muscles from pigs that display susceptibility to stress can negatively affect the quality of meat that they produce by increasing the amount of metabolic activity. This results in a high level of lactate produced in a short amount of time and thus, creating a very rapid pH decline while the carcass temperature is still high. In order to simulate the effects of stress

susceptibility, malignant hyperthremia susceptible (MHS) and malignant hyperthermia resistant (MHR) pigs have been studied to determine the effect of stress on Ca^{2+} release and uptake to the sarcoplasmic reticulum. MHS and MHR pigs were determined by DNA analysis of blood samples.

Kuchenmeister and others (1999a) studied the effects of the MHS and MHR genotypes have on pork quality and cell injury. Pigs that were MHS had higher L* values, higher drip loss, and lower pH at 45 minutes postmortem. No significant difference in ultimate pH was observed between the two genotypes. It was also observed that the permeability of the sarcoplasmic reticulum membranes to Ca^{2+} was already disturbed at 45 minutes and 4 hours postmortem in MHS susceptible pigs, displaying a greater permeability and greater Ca^{2+}-ATPase activity at these time points for MHS pigs. This suggests that MHS pigs are experiencing more Ca^{2+} being released into the cell and the cell is trying to maintain proper Ca^{2+} level in the cell by increasing the activity of Ca^{2+}-ATPase to remove Ca^{2+} back to the sarcoplasmic reticulum. Another study by Kuchenmeister and others (1999b) displayed that the ability of MHS pigs to sequester Ca^{2+} release declined 40% in MHS pigs compared to 5% in MHR pigs at 45 minutes postmortem. This study also displayed that the ryanodine receptor is functional up to 22 hours postmortem and this channel is nearly fully open in MHS pigs at 0 minutes, 45 minutes, 4 hours, and 22 hours postmortem. The Ca^{2+} release channel is only partially opened in the MHR samples at the aforementioned time points. Determining the status of the channel was determined with incubation of the ryanodine receptor with ryanodine, a potent inhibitor of the ryanodine receptor that effectively closes the Ca^{2+} release channel.

The effects of season can also affect the ability of the sarcoplasmic reticulum to regulate Ca^{2+}. Kuchemeister and others (2000) used 2 batches of MHS and MSR pigs and slaughtered one batch of each during June and July and the remaining batches in November and December. In both the MSR and MHS pigs, the regulation of Ca^{2+} release and uptake was reduced in the summer months. The pigs harvested in the summer months also displayed poorer quality attributes in terms of pH at 45 minutes postmortem, color, and drip loss (Kuchenmeister et al., 2000).

Proteolysis of Cytoskeletal Proteins by Calpains

Nomenclature and Function of Calpains

The calpain family is a superfamily of multi-domain proteases which have a common catalytic calpain domain (Domain II) and which also have some resemblance to the classical papain family (Reverter et al. 2001). The calpains are ubiquitous proteases found in the cytosol of all living cell tissues. The most characterized of the calpain family are: μ-calpain and m-calpain, and their naturally occurring inhibitor, calpastatin (Goll et al. 1999). The primary structure reveals 50-60% sequence homology between μ- and m-calpain (Goll et al., 1991). The nomenclature for μ- and m-calpain comes from the different amounts of Ca^{2+} that is required to activate the proteases in vitro (Suzuki, 1991). μ-Calpain requires 5-70 μM Ca^{2+} in vitro in order to activate it to half maximal activity whereas m-calpain requires 100-2000 μM Ca^{2+} for half maximal activity (Goll et al., 1992). In general, skeletal muscle in mammals contains equal amounts of μ-calpain and m-calpain (Goll et al. 1999).

The function of the calpains is not known exactly, although there is much evidence suggesting that the calpains contribute to many cellular processes. These processes include

signal transduction, apoptosis, cell cycle regulation, and cytoskeletal reorganization (Hosfield et al., 2001). Excessive proteolysis of the calpains has been observed in several neuropathological disorders such as Alzheimer's disease (Hosfield et al., 2001). Although little is known about skm-calpain, defects in skm-calpain have been linked to the development of limb girdle muscular dystrophy 2A (Hosfield et al., 2001).

Immunolocalization has shown that the calpains and calpastatin are found intracellularly (Goll et al. 1992). The calpains and calpastatin can be located near the Z-disk, with smaller amounts in the I-band and in the A-band area (Goll et al. 1999).

The calpains degrade most but not all cytoskeletal proteins found in the muscle. The calpains have been shown to degrade desmin (O'Shea et al., 1979), nebulin (Taylor et al., 1995), talin (Hemmings et al., 1996), titin (Suzuki et al. 1996), troponin T (Ho et al., 1994), and vinculin (Taylor et al., 1995). However, the calpains do not cleave actin (Goll et al., 1991) or myosin (Pemrick and Grebanau, 1984), which are the main myofibrillar proteins. It has been shown that the 200-kD myosin is slowly "nibbled" at its N-terminal end by the calpains to produce a degradation product of polypeptides at the 150-180-kD range (Pemrick and Grebanau, 1984). Calpains also do not degrade collagen (Goll et al. 1999).

The ryanodine receptor is also susceptible to degradation by calpains. Gilchrist and others (1992) observed the production of an approximately 410 kDa degradation product and also an approximately 150 kDa degradation product when exposed to μ-calpain and m-calpain. Iino and others (1992) reported similar results when they observed ryanodine receptor incubation with m-calpain resulted in an approximately 430 kDa polypeptide that was further cleaved to an approximately 150 kDa polypeptide. It is also interesting to note that even after most of the ryanodine receptor was cleaved by m-calpain, the samples

retained their Ca^{2+} sensitivity, although the maximum rate of Ca^{2+} release doubled in samples that were incubated with m-calpain (Iino et al., 1992). The authors suggested that the N-terminal region may suppress the activity of the ryanodine receptor when the channel is intact (Iino et al., 1992).

Shoshan-Barmatz and others in 1994 observed similar degradation patterns of the ryanodine receptor and also observed that maximal degradation of the ryanodine receptor by μ-calpain (based on Ca^{2+} activity assays) occurred at pH 7.0-7.5. Also, it was observed that incubation with ATP and high NaCl concentrations strongly inhibited the degradation of the ryanodine receptor.

m-Calpain has also been shown to effectively degrade the ryanodine receptor. Brandt and others (1992) observed that m-calpain degrades the ryanodine receptor in both the skeletal muscle triad junctions and in the purified form of the ryanodine receptor. The cleavage of the 565 kDa monomer led to the production of a 160 and 410 kDa peptides. The 160 kDa peptide is stable but the 410 kDa peptide was degraded to 70 and 340 kDa peptides. This 70 kDa fragment was also predicted to be in the C-terminal region of the ryanodine receptor. This C-terminal region is believed to be the region that spans the membrane. m-Calpain proteolysis of the ryanodine receptor was also found to be inhibited by calmodulin. In compiling known calpain substrates, it has been noted most of these proteins contained both calmodulin binding sites and PEDST regions (Wang et al., 1989). PEDST regions are hydrophilic segments that are rich in proline, glutamate, aspartate, serine, and threonine and are also devoid of any basic amino acids. These PEDST domains have been hypothesized to be recognizers for calpains (Rogers et al., 1986). The importance of the calmodulin binding sites is that occupation of the calmodulin binding site by calmodulin altered or inhibited

calpain degradation that usually occurred at or near the calmodulin binding site (Wang et al., 1989). Brandt and others (1992) were able to identify eight PEDST sites on the ryanodine receptor. The combined observations of the PEDST sites and calmodulin inhibition suggest that calpain-degraded fragments of the ryanodine receptor start and/or end in the calmodulin binding regions of the ryanodine receptor.

The sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump has been shown to be a substrate for calpains. Wang and others (1988b) displayed that incubation of purified SERCA with μ-calpain did degrade the SERCA, resulting in two degradation products at approximately 85 kDa and 82 kDa. It was then shown that degradation of the SERCA by μ-calpain resulted in an irreversible activation of the SERCA (Wang et al., 1988a). It was suggested by the authors that this irreversible activation might be a last defense mechanism for the cell to be able to remove large amounts of Ca^{2+} from the cell (Wang et al., 1988a). A study by Seidler and others (1998) observed that cardiac SERCA was susceptible to degradation by calpain. Incubation with calpain degraded SERCA to 75.8% of its control when 3.4 units calpain/mL and 0.8 mg cardiac sarcoplasmic reticulum were incubated for 30 minutes at 30°C.

Structure and Activation of Calpains

The first purification of a protein belonging to the calpain system occurred in 1976 when Dayton and others purified m-calpain from porcine skeletal muscle (Dayton et al. 1976). Mellgren and others were then able to identify μ - calpain as being a part of the calpain family, leading to its purification in porcine skeletal muscle by Dayton in 1981 (Goll et al 1999).

The typical calpains consist of a large subunit that migrates at 80 kDa and a small subunit that migrates at 28 kDa. The primary structure of μ-calpain consists of 714 amino acids in the large subunit (Aoki et al., 1986) and 268 amino acids in the small subunit (Ohno et al.,1986). μ-Calpain and m-calpain 80 kDa subunits are different polypeptides, but share 50-60% sequence homology (Goll, 1991). The 28 kDa subunit of both μ-calpain and m-calpain are identical.

The primary structure of either μ- or m-calpain predicts four domains. Domain I (dI) is the N-terminal domain that contains the first approximately 90 amino acids of the polypeptide (Goll, 1991). This domain comprises of a single α-helix that is anchored in a cavity of domain IV (Khorchid and Ikura, 2002). This anchoring then stabilizes the circular domain arrangement of calpain.

Domain II (dII) consists of approximately amino acids 91-300. Domain II is also the domain that contains a cysteine residue that is considered to be part of the active site of the enzyme (Goll, 1991). This domain is homologous to papain, but shares very little sequence homology with any other cysteine proteases. Domain II can also be separated into two sub domains known as domain IIa and domain IIb. These sub domains have been shown to form a catalytic triad that is similar to other cysteine proteases, such as papain (Khorchid and Ikura, 2002). This triad in μ-calpain consists of His 262, Cys 105, and Asn 268 (Khorchid and Ikura, 2002).

Domain III (dIII) is from approximately amino acid 300-500 and no sequence homology is observed between this amino acid sequence and any other protein (Goll, 1991). Domain III consists of eight strands that are similar to the C2 domain found in various proteins such as protein kinase C and phospholipase C (Khochid and Ikura 2002). This

domain also contains an acidic loop that has been suggested to play a role in the Ca^{2+} activation of calpain (Hosfield et al., 1999; Strobl et al., 2000).

Domain IV (dIV) is from approximately amino acid 500-700. This domain contains an amino acid sequence that is homologous to calmodulin and also contains five sequences analogous to the E-F hand Ca^{2+}-binding sequences (Goll, 1991). Domain IV, along with domain VI is responsible for the heterodimerization of the large and small subunits through their interaction between their fifth E-F hand motifs (Khorchid and Ikura, 2002).

The small subunit primary sequence predicts two domains. Domain V is largely unresolved in its crystal structure but does contain a glycine rich region in its N-terminal region (Khorchid and Ikura, 2002). This glycine rich region partakes in the hydrophobic nature of the domain (Sorimachi and Suzuki, 2001). Domain VI works with domain IV as Ca^{2+} binding domains.

Calpain molecules undergo autolysis as a direct result of calpain molecules degrading other calpain molecules. The rate of μ-calpain autolysis is directly related to Ca^{2+} concentration. Cottin and others (2001) observed that autolysis of μ-calpain is very rapid at 5 mM Ca^{2+}, less rapid at 2mM Ca^{2+}, and even slower at 100μM Ca^{2+}. The result of autolysis to the 80 kDa μ-calpain molecule is a removal of a 14 amino acid sequence from the N-terminus. This yields an active 78 kDa intermediate product. When this occurs, 12 more amino acids are cleaved from the μ-calpain N-terminus of the 78 kDa subunit to yield an active 76 kDa subunit (Zimmerman and Schlapfer, 1991).

Moldovenu and others have explained Ca^{2+} binding to calpains more thoroughly since the findings of the two Ca^{2+} binding sites in two subdomains of domain II in 2002. These two subdomains are part of what is referred to as mini-calpain. This region is only

catalytically active upon loading of Ca^{2+}, and tryptophan fluorescence demonstrated that Ca^{2+} binding involves multiple sites (Khorchid and Ikura, 2002).

As revealed in its crystal structure, the Ca^{2+} binding site in domain IIa of μ-calpain involves residues Asp 106, Val 99, and Gly 101 on the loop preceding α-helix 3 (Khorchid and Ikura, 2002). This α-helix contains the catalytic cysteine residue, Cys 115. Ca^{2+} binding also involves Glu 185, which is positioned on loop leading to the N-terminus of the core of α-helix 5 (Khorchid and Ikura, 2002).

In domain IIb of μ-calpain, the Ca^{2+} binding site is coordinated by Glu 302 and the Asp 309 that are a part of the loop containing Trp 298, an amino acid residue that is part of the catlytic active site (Khorchid and Ikura, 2002). Ca^{2+} binding also involves residues Asp 331, Glu 333, and Met 329 in the loop between α-helices eight and nine (Khorchid and Ikura, 2002).

The active site of calpain involves the location and spacing of several critical active site residues: Cys 115, His 272, Asn 296, Gln 109, and Trp 298 (Khorchid and Ikura, 2002). These active site residues resemble the active site observed in other cysteine proteases.

There is a relatively small backbone conformational change between bound and Ca^{2+}-free calpains, however there is a major change that occurs within two loop regions of domain IIa (Gln 96-Cys108) and domain IIb (Ile 254-Val 269) (Khorchid and Ikura, 2002). In the presence of Ca^{2+}, residues in Ile 254-Val 269 loop form a β-sheet that provides van der Waals attractions to the active site on Trp 298 (Khorchid and Ikura, 2002). The residue Trp 298 then acts as a wedge between domain IIa and domain IIb. The sidechain of Trp 298 then moves away from the active site cleft, allowing for the proper formation of the catalytic triad: His 262, Cys 105, and Asn 286 (Khorchid and Ikura, 2002). Although this region is similar

to other cysteine proteases, the nature of domain IIa and domain IIb show that activity is a structural phenomenon, not a pro-peptide inhibition that is found in other cysteine proteases such as the cathepsins (Sorimachi and Suzuki, 2001).

In addition to domain II, Ca^{2+} is bound at the EF hands in domains IV and VI. In order to explain its activation, a two-step mechanism has been proposed by Moldovenu and others (2002). The first step requires a release of the constraints imposed by the circular arrangement of the domains. This would result in subtle conformational changes in domains IV and VI when bound to Ca^{2+}. This would then lead to a relaxation of the interaction between the N-teminal α-helix of domain I and the second EF hand motif of domain VI. This may possibly promote the cleavage of the large subunit from the small subunit. The second step would involve the realignment of the active site cleft by the cooperative binding of Ca^{2+} to domain IIa and domain IIb. This two-step mechanism would add an extra layer of control over calpain function by requiring Ca^{2+} to be bound to both domain II and the EF hands of domains IV and VI.

Is μ-Calpain or m-Calpain Active During Postmortem Conditions?

In order for a protease to be active during postmortem aging, it must be active when faced with the environmental conditions of postmortem tissue and storage of the product. Some of the obstacles facing whether or not m-calpain is active during postmortem conditions is that m-calpain is maximally active at pH 7.5 (Dayton et al., 1976), thus having minimal activity at the pH that is generally attained in post mortem muscle (pH ~ 5.5). Furthermore, the Ca^{2+} concentration required for activation of m-calpain exceeds the level of

Ca^{2+} that is attained in postmortem muscle. Based on these findings, it would seem unlikely that m-calpain is active during the conditions present during postmortem storage.

In 1986, Koohmarie and others conducted research to answer if μ-calpain is active during postmortem conditions. It was then discovered that based on SDS-PAGE and electron microscopy, μ-calpain retains 24%-28% of its activity at pH 5.5-5.8 and at 5°C. This level was also sufficient to reproduce the changes in the myofibrils associated with postmortem storage. This study was able to show that μ-calpain could be active at the conditions encountered during postmortem storage, thus giving rise to the thought that μ-calpain is the primarily responsible protease of the calpains family involved in postmortem proteolysis.

Koohmarie and others in 1988(a) showed that the calpains are capable of reproducing the majority of changes that are associated with aged meat. This was observed by measuring the μ-calpain and m-calpain activity from the longissimus dorsi, biceps femoris, and psoas major. The activity was compared to the aging response of these three muscles, indicating that initial levels of μ-calpain activity may determine the aging response of a specific muscle.

The effects of postmortem storage on the activities of μ-calpain and m-calpain have also been studied. The activity of m-calpain remained nearly constant throughout postmortem aging, while there was a steady decrease in the activity of μ-calpain (Koohmarie et al. 1987, 1988, 1992), further suggesting that μ-calpain may be responsible for postmortem proteolysis as opposed to m-calpain. The rationale for this observation stems from the idea that both μ-calpain and m-calpain undergo autolysis and subsequent activation of the protease. This activation continues upon further autolysis if the Ca^{2+} concentration is

sufficient for activation of the protease (Koohmarie, 1988). Therefore, the loss of μ-calpain activity (Koohmarie et al., 1987) during postmortem storage may be a good indicator that μ-calpain is active under postmortem conditions, not m-calpain.

Videlenc and others (1983) also studied the effects of Ca^{2+} concentrations. It was discovered that both μ-calpain and m-calpain were autolyzed when in the presence of a concentration of 3 mM Ca^{2+}. However, at 50 μM Ca^{2+} only μ-calpain was activated. This Ca^{2+} concentration is a concentration that can be reached by the muscle cells during postmortem storage (Goll et al. 1983). This finding further suggests that it may be μ-calpain that is active during postmortem storage and not m-calpain.

As a further study of the activity of μ-calpain activation under postmortem conditions, Huff-Lonergan and others (1996) observed myofibril degradation by μ-calpain at pH 5.6, 4°C, and 100 μM $CaCl_2$. At these conditions it was observed that myofibrils that were incubated with purified μ-calpain showed degradation of the myofibrillar proteins titin, nebulin, desmin, and troponin-T. By showing degradation of these myofibrillar proteins when incubated with μ-calpain during simulated postmortem conditions, this study added further evidence to the hypothesis that μ-calpain is responsible for postmortem proteolysis of myofibrillar proteins. This study by Huff-Lonergan and others (1996) indicated that an increased level of postmortem tenderization occurred in samples that had increased titin, nebulin, desmin, and troponin-T degradation of the intact proteins.

Intact desmin may be an indicator of tenderness. Desmin has been shown to be susceptible to proteolysis by μ-calpain (O'Shea et al., 1979, Huff-Lonergan et al., 1996). Since desmin may function to maintain the integrity of muscle cells, its degradation may

compromise the structure of the muscle cell (Robson, 1995). This effect of desmin on postmortem tenderness has been studied. It has been observed that samples that have increased tenderness have also been observed to have more desmin degradation (Koohmaraie et al., 1984; Ho et al., 1996; Huff-Lonergan et al., 1996; Rowe et al., 2001)

Boehm and others (1998) conducted a study that investigated the changes of calpains and calpastatin in bovine muscle. Using SDS PAGE, m-calpain was not found to be autolyzed during postmortem storage of up to 7 days. This would suggest that there is a relatively minimal change in the activity of m-calpain during postmortem storage. The Ca^{2+} level required for activation of m-calpain could however be sufficient for activation in the time postmortem. Parrish and others (1981) found the Ca^{2+} concentration in postmortem muscle (at 10-14 days post mortem) to be 630-970 μM. This increase in the Ca^{2+} concentration could be attributed to the costameres and muscle sarcolemma becoming degraded, thus allowing a leakage of Ca^{2+} into the muscle cell (Taylor et al., 1995). This free Ca^{2+} in the cell after degradation of the sarcolemma is well within the range that is required for Ca^{2+} activation of m-calpain. It could then be possible that m-calpain is responsible for proteolysis at later time points post mortem.

It has also been observed that μ-calpain becomes more associated to the myofibril as time postmortem increases (Boehm et al., 1998). Most of this myofibril bound μ-calpain is autolyzed to the 76 kDa form at one day postmortem and all of the myofibril bound μ-calpain is in the 76 kDa form at 7 days post mortem. It could then be reasoned that its Ca^{2+} concentration for half maximal activity is 0.5 to 2 μM (Goll et al., 1995), based on its autolysis to its 76 kDa form. This Ca^{2+} concentration is well below the 100-970 μM free

Ca^{2+} that is reported to exist in postmortem muscle (Parrish et al., 1981). However, this study done by Boehm and others (1998) observed that there wasn't any substantial proteolysis observed from the myofibril bound μ-calpain.

Delgado and others in 2001 observed the properties of myofibril-bound calpain in longissimus muscle from both callipyge and noncallipyge sheep at 0, 1, 3, and 10 days of postmortem storage at 4°C. This study observed that myofibril bound calpain activity increased by 56% to 74% during the first day of storage from its initial calpain activity. Western blot analysis of myofibrils that had been incubated with purified μ-calpain indicated that titn, nebulin, and desmin were degraded in the presence of Ca^{2+}. It was also observed that this myofibril bound calpain was only partially susceptible to inhibition by E-64, leupeptin, indoacetate, and calapstatin. This study would suggest that myofibril-bound calpain is indeed active, even in the presence of known inhibitors. The nature of this activity still remains unclear.

Factors That Affect Calpain Activity

Several factors can influence the activity of the calpains. One factor is that of pH. Rowe and others (2001) observed that there were differences observed between pH, extent of autolysis, and the amount and rate of degradation of key myofibrillar and cytoskeletal proteins. It was observed, using western blot analysis, that the porcine longissimus dorsi (LD) that displayed the lowest pH at 2 hours postmortem had the least detectable intact 80 kDa band and also had the least detectable activity at 24 hours postmortem, using casein zymography. These samples showed no μ-calpain activity at 72 hours. Porcine LD samples that were observed to have higher pH values at 2 hours postmortem (greater than 5.8) had

less extensive autolysis than samples that had a lower pH. This also corresponded to the samples with higher pH having some activity of μ-calpain at 72 hours. However, it was also observed that samples that had a low pH at 2 hours postmortem had the earliest degradation of troponin-T and desmin. This would suggest that slightly lower pH values at early times postmortem might accelerate the activity of μ-calpain but may not be an indicator of the extent of μ-calpain activity. A study done by Melody and others (2003) found significant differences in pH at 45 minutes postmortem between three muscle types. The psoas major had a significantly ($P < 0.0001$) lower pH at 45 minutes postmortem than either the longissimus dorsi or semimembranosus. Western blot analysis at 45 minutes postmortem revealed that the psoas major had more extensive autolysis than the other two muscles and also had more myofibril-bound μ-calpain at 45 minutes postmortem than either the LD or the SM.

Ionic strength is another factor that affects the stability and thus activity of μ-calpain. It was observed that as ionic strength increases, the activity of the autolyzed μ-calpain decreased, despite its apparent stability as determined by anion exchange chromatography (Geesink and Koohmarie, 2000). μ-Calpain's stability was determined by using SDS-PAGE. At 25°C, nearly maximal activity was observed at 50 mM NaCl through 120 minutes. The activity of μ-calpain began to decrease as ionic strength was increased. The ionic strength in postmortem muscle is equivalent to 0.2 to 0.3 M NaCl (Winger and Pope, 1980-81). This data would suggest that an environment with a high ionic strength would inhibit the activity of μ-calpain.

Calpastatin, the naturally occurring inhibitor of the calpains, has been found in all tissues that contain calpains and is believed to play a major role in the regulation of proteolysis that occurs as a result of the calpains (Huff-Lonergan and Lonergan, 1999). Its primary structure can be defined by four repeating subunits that contain homologous amino acid sequences of roughly 140 residues and a unique alkaline N-terminal region known as domain L (Maki et al., 1991). Although it is unknown what the precise mechanism for calpastatin inhibition, it is hypothesized to inhibit calpains by binding to domains IV and VI (Nisimura and Goll, 1991) of μ-calpain. In general, the interaction of calpastatin to the calpains is reliant on Ca^{2+} concentration, as Ca^{2+} is required to allow calpastatin to bind to the calpains (Kapprell and Goll, 1989). This reaction is also reversible by the addition of Ca^{2+} chelators (Maki et al., 1990). In autolyzed μ-calpain, less Ca^{2+} was required for half maximal binding to calpastatin for activity (Kapprel and Goll, 1989). Unautolyzed μ-calpain has a higher Ca^{2+} requirement for half maximal binding to calpastatin (Kapprell and Goll, 1989). It has also been observed that calpastatin was not able to inhibit the initial autolysis step of the 80 kD subunit, but calpastatin was able to inhibit calpain autolysis from 78 kD to 76 kD (Zimmerman and Schlaepfer, 1991).

As previously discussed, calpains have the ability to degrade specific intermyofibrillar, costameric, and myofibrillar proteins during postmortem storage. Degradation of an intermediate filament protein such as desmin may have an important role in the water-holding capacity and tenderness of the product.

Water-Holding Capacity and Tenderness

Water-Holding Capacity of Fresh Meat

Water-holding capacity is a very important attribute of meat products, both from a quality and economic standpoint. Water-holding capacity can be defined as the ability of meat to retain its water despite the application of force (van Laack, 1999). From a meat processor's view, the ability of a product to retain water means added weight to the product, which in turn increases the economic value of the product. Furthermore, a product's ability to retain water also plays a role in consumer acceptance of the product. A product that has a low water-holding capacity will have adverse affects in terms of appearance such as the appearance of excess purge in the retail case or a dry appearance to the product. A product with a low water-holding capacity also negatively affects sensory characteristics such as mouth-feel and juiciness.

At the time of slaughter, muscle contains about 75% water (van Laack, 1999). A majority of this water is found in the intracellular portion of the muscle, between the thin and thick filaments with the remaining water found in the extracellular spaces (Hamm, 1972 as cited by van Laack, 1999.). Since 80% of the muscle fiber is made up of myofibrils, it can be deducted that myofibrils constitute the largest compartment for water in the muscle fiber (Offer and Knight, 1988). Charged areas of amino acids that are part of the proteins allow for an interaction between the protein and the strong dipolar water molecule.

The water within the cell can be divided into three different states: constitutional water, interfacial water, and bulk water (Hamm, 1986). Constitutional water is water that is directly bound to the protein and is actually found within the protein. Constitutional water comprises a very small portion of the total water in the cell. This protein-bound form of

water constitutes less than 0.1% of the total tissue water. Interfacial water is water that is attracted to the constitutional water at the surface of the proteins. Interfacial water is immobilized due to the strong attraction of the interfacial water to the protein-bound water. Bulk water can also be considered to be "free" water within the cell. This water also undergoes water-water interactions, however these interactions are less strong, allowing for the possibility of this water to become lost to the protein.

Extracellular water is also present in muscle tissue. The swelling and shrinking of the cell is a result of the balance of intracellular and extracellular water. Swelling of the cell can be caused by an increase in the movement of extracellular water into the cell, causing an increase in intracellular water (Hamm, 1986). Shrinking of the cell can be caused by an increase in the movement of intracellular water outside of the cell (Hamm, 1986). Several factors can influence this balance between intracellular and extracellular water and also can potentially influence drip loss. These factors are genotype, pre-slaughter stress and stunning method, method of cooling the carcass post-mortem, rate of pH decline, and ultimate attained pH, and proteolysis. The factors discussed in this review of the literature will be rate of pH decline, ultimate attained pH, and proteolysis.

Factors That Can Influence Water-Holding Capacity

Rate of pH decline is a factor that can influence water-holding capacity. The driving force behind the rate of pH decline is the rate of postmortem glycolysis. A possible result of the high rate of glycolysis in post-mortem muscle tissue may be an increased amount of lactate produced, thus lowering the pH of the product. In a study conducted by Lonergan and others (2001), it was observed that pigs that were selected for lean growth efficiency had a

significantly lower pH decline from the control genetic line. The pigs that displayed a rapid pH decline also displayed a greater amount of drip loss in the longissimus dorsi (1, 3, and 4 days storage), semitendinosis (2, 3, and 4 days storage), and semimembranosus (1, 2, 3, and 4 days storage).

This rapid pH decline can cause protein denaturation while the carcass temperature is still high (Offer and Knight, 1988). This denaturation of contractile proteins causes the length between actin and myosin to shrink due to the length of the myosin heads decreasing from 19 nm to 17 nm (van Laack, 1999). This small change in the length of these crossbridges can cause the filament spacing to decrease, resulting in more intracellular water to be expelled into the extracellular spaces. Extreme denaturation of major contractile proteins such as myosin (Penny, 1969) can cause major texture and water-holding capacity problems in the product, commonly known as a pale, soft and exudative (PSE) product.

Another factor that plays a role in determining the water-holding capacity of a product is the ultimate pH attained by the carcass post-mortem. Typically, the pH of a normal pork longissimus dorsi is around 5.5-5.6. This ultimate pH is important in terms of the isoelectric point of many of the major proteins found in meat. The isoelectric point can be defined as the pH at which there is no net charge on the protein. One of the major proteins found in meat is myosin, which has an ioselectric point of about 5.1. As the pH becomes closer to 5.1, there is less of a net charge on the protein, thus reducing the ability of the protein to bind to the dipolar water molecule. Another effect of an ultimate pH that is close to the isoelectric point of many of the proteins in meat is that there would be less space between the thick and thin filaments due to less charge for repulsion of the proteins.

Proteolysis of certain cytoskeletal proteins can also affect water-holding capacity. Kristensen and Purslow (2000) proposed that degradation of the cytoskeleton of the muscle fiber during ageing might increase water-holding capacity by removing inter-myofibrillar and costameric connections. This would reduce or possibly remove the rigor-induced lateral shrinkage of myofibrils and the shrinkage of the whole muscle fiber, resulting in an inflow or possibly a lack of outflow of intracellular water to the extracellular compartment (Kristensen and Purslow, 2000). In agreement with this hypothesis, degradation of the cytoskeletal protein desmin has been shown to correlate with reduced drip loss in pork products (Rowe, et al., 2001). In another study done by Melody and others (2003), three different muscle types were examined. It was observed that the psoas major had the least amount of drip loss and also had more desmin degradation than both the longissimus dorsi and semimembranosus at 45 minutes postmortem. This suggested that less water is lost initially resulting in a greater water-holding capacity.

Factors That Can Influence Tenderness

The perception of tenderness by the eater can be described as sensations effected by certain conditions within the meat during mastication (Aberle et al., 2001). These conditions include softness to tongue and cheek, resistance to tooth pressure, ease of fragmentation, the sensation of mealiness and adhesion, and the presence of a residue after chewing (Aberle et al., 2001). Several factors that can affect these tenderness sensations are sarcomere shortening, pH decline of the carcass, extent of proteolysis, activity of calpastatin, and amount of collagen present in the product.

Cold shortening and thaw rigor are conditions that occur as a result of temperature abuse of the carcass that leads to extremely short sarcomeres. Cold shortening develops as a result of the carcass being chilled prior to the full development of rigor, which relates to a large release of Ca^{2+} into the sarcoplasm, causing a severe muscle contraction (Aberle et al., 2001). Thaw rigor is a more severe case that develops as a result of muscle that was frozen prerigor is thawed, causing a large release of Ca^{2+} into the sarcoplasm and a severe muscle contraction that can result in a physical shortening of up to 80% of the original sarcomere length (Aberle et al., 2001). Both cold shortening and thaw rigor result in an increase in the densities of the myofibrils, thus decreasing tenderness. Koohmaraie and others (1996) observed the effects of clamping ovine losgissimus muscles at each end to prevent sarcomere shortening in times postmortem up to 24 hours. This study observed that shear forces were statistically the same at 0 hours (4.5 kg) and at 24 hours (4.9 kg) postmortem (Koohmaraie et al., 1996), suggesting that meat toughening in ovine muscles that occurs in the first 24 hours postmortem could be due to sarcomere shortening.

A more rapid pH decline has been shown to negatively affect tenderness. A study done by Taylor and Perry (1995) used electrical stimulation of pork carcasses at 20 minutes post-harvest to observe its effect on pork quality attributes. Pigs that had been electrically stimulated had significantly lower pH values at 3 hours postmortem, suggesting a more rapid pH decline (Taylor and Perry 1995). Tenderness was also observed to be significantly lower in the electrically stimulated samples as well, with stimulated muscles having tenderness values of 4.79 kg and the non-stimulated samples having tenderness values of 3.41 kg after 10 days of storage (Taylor and Perry 1995). Lonergan and others (2001) noted similar results when selecting pigs for lean growth efficiency and observing differences between this line of

pigs and a control line. The pH of the longissimus was found to be significantly lower in the lean growth line at 15, 30, and 45 minutes postmortem than the control line (Lonergan et al., 2001). Warner-Bratzler shear analysis also revealed differences in tenderness between the two lines as the lean growth line had WBS value of 3.12 kg and the control line had a WBS value of 2.62 kg (Lonergan et al., 2001).

An increase in the extent of protein degradation in muscle postmortem has been related to increase tenderness. Proteolysis results in ultrastructrual changes in the muscle structure in times postmortem. Increased proteolysis of specific structural proteins by μ-calpain is related to increased tenderness. A study by Taylor and others (1995) used electron microscope imaging to observe the possible degradation of the Z-disk. This study observed that during the firs 3 or 4 days of storage at 4°C, costmeric proteins (desmin, vinculin, and dystrophin) and N_2 lines (titin and nebulin) are degraded. The costameric proteins serve to attach the sarcolemma to the myofibril and the N_2 line which extends through the entire I-band and anchors into the Z-disk (Taylor et al., 1995) suggesting that an increase in the degradation of these proteins may be related to increased tenderness. Huff-Lonergan and others (1996) observed that beef samples that displayed significantly lower shear force values at 1 day postmortem also displayed faster degradation of titin, nebulin, filamin, desmin, and troponin-T after incubating purified myofibrils with purified μ-calpain.

Calpastatin is the natural inhibitor for the ubiquitous calpain proteases and is believed to play a major role in the regulation of proteolysis that occurs in the time postmortem (Huff-Lonergan and Lonergan, 1999). The effect of different levels of calpastatin has on μ-calpain activity was studied by purifying μ-calpain and calpastatin from bovine muscles. Aliquots of purified myofibrils were then incubated with 0.12 units of μ-calpain and 0, 0.25, and 0.50

units of calpastatin for 0, 1, 2, 7, and 14 days at 5°C. This study observed that increasing the amount of calpastatin limited both the rate and extent of proteolysis of the purified myofibrils but did not completely inhibit proteolysis (Geesink and Koohmaraie, 1999). Extent of proteolysis was determined by the presence and degradation of titin, myosin heavy-chain, α-actinin, desmin, actin, and troponin-I. Another study by Lonergan and others (2001) measured calpastatin activity in pigs from a lean growth selected line and a control line. Calpastatin activity in the pigs selected for lean growth efficiency had significantly higher calpastatin activities (3.38 units/g tissue) than the control line (2.98 units/g tissue) (Lonergan et al., 2001). The line selected for lean growth efficiency also displayed significantly less Troponin-T degradation, suggesting that an increase in calpastatin activity results in less proteolysis in the muscle (Lonergan et al., 2001).

Collagen is a white fibrous connective tissue protein that in high amounts and when chemically mature, can negatively influence tenderness (Aberle et al., 2001). Chemically mature collagen that is formed as an animal's age increases and also forms many heat stable cross-links that retain high residual strength after cooking, yielding a less tender product (Aberle et al., 2001). A study by Wheeler and others (2002) found that a trained sensory panel analysis for tenderness in cooked product was significantly correlated to the amount of collagen present in the sample ($r = -0.45$) but was found to have an insignificant correlation in pork ($r = -0.12$). An earlier study by Wheeler and others (2000) observed a significant correlation between pork tenderness at 24 hours postmortem and the amount of collagen present ($r = -0.34$), suggesting that as tenderness increases the amount of collagen decreases.

As previously discussed, many factors such as pH decline, proteolysis, and other environmental factors can contribute to the quality attributes of meat products. Another

important environmental factor that needs to be discussed is the effect of oxidation on Ca^{2+} regulating proteins and the calpains system and how oxidation and antioxidant supplementation may affect meat quality.

Effect of Oxidation on Meat Quality

Oxidation in Aerobic Organisms

Around 2-3 billion years ago, oxygen was introduced into the earth's atmosphere by the evolution of organisms undergoing photosynthesis and releasing oxygen as a byproduct. Within a few million years, oxygen made up close to 21% of the oxygen in the atmosphere (Sen and Packer, 2000). In humans, 65% of the body is made up of oxygen, mostly coming from water. This abundance of oxygen in the body allows for processes such as oxidative metabolism occur. This involves the combustion of glucose and lipids to be used for energy. As the earth evolved into an oxygen rich atmosphere, antioxidant defense systems were also evolving to protect systems from reactive oxygen and nitrogen species.

Aerobic organisms derive their energy from the oxidation of fuel molecules such as glucose or fatty acids. The process of oxidation involves an electron from these fuel molecules and that electron is transferred through a series of reactions to reach an electron acceptor. In this case, the electron acceptor is O_2. O_2 has 2 unpaired electrons in its π^* molecular orbital. If an electron is added to O_2, it enters one of these π^* orbitals. This then yields a superoxide radical (O_2^{*-}) with only one unpaired electron. The addition of one or more electrons to the superoxide radical results in the formation of the non-radical species, peroxide (O_2^-), which forms hydrogen peroxide (H_2O_2) in the presence of protons.

Oxidized metals such as Fe^{3+} and Cu^{2+} can catalyze electron transfer from one oxygen species to another. Reduced metal ions such as Fe^{2+} and Cu^{+} can donate one electron and start a sequence of reactions that can end in the formation of superoxide radicals. An example of such a reaction is the Fenton reaction where the very reactive hydroxyl radical (*OH) is formed by a series of reactions involving Fe^{2+} and H_2O_2.

In aerobic organisms, metals catalyze almost all steps in the reduction of O_2. Incorporating the metal into the enzyme controls, a majority of the time, the reactivity of the metal. Some examples of enzymes where this occurs would be superoxide dimutase, catalase, and cytochromes. These enzymes are all examples of highly effective antioxidant systems within the cell (Butterfield et al., 1997).

Unfortunately, these reactions yield the formation of reactive oxygen species (ROS). Examples of ROS are H_2O_2 and OH as well as many others. These molecules can damage other biomolecules such as DNA, proteins, and lipids. Mitochondria are the major source of free radicals intracellularly as the electron transport system of the mitochondria that is used for energy production gives rise to these ROS.

When compounds with one or more unpaired electrons are small and freely diffusible, they are referred to as free radicals. ROS such as O_2, O_2*-, and *OH and certain metal ions with unpaired electrons in their *d* orbitals are all considered free radicals. In this way, each reactant (ROS and Fe^{3+} for example) donates one electron to the new bond.

In lipid oxidation, the formation of free radicals involves three steps: initiation, propagation, and termination (Burton and Traber, 1990). Initiation involves the production of carbon centered lipid radicals (R*). These radicals react very rapidly with oxygen to form a peroxyl radical (ROO*), which is the hallmark of propagation. This peroxyl radical is a

chain carrying radical that can attack other unoxidized polyunsaturated lipid molecules. Although, the initial reaction yields a hydroperoxide (ROOH), the second reaction also produces another R*. This then becomes a runaway process that consumes valuable polyunsaturated lipids and produces a corresponding amount of ROOH.

Propagation: $R^* + O_2 \longrightarrow ROO^*$ (fast)

$ROO^* + RH \longrightarrow ROOH + R^*$

The final step, termination, occurs when a chain carrying peroxyl (ROO*) combines with another chain carrying peroxyl (ROO*) to form inactive products. This then terminates the oxidation reaction as the reaction has somewhat "consumed" itself out.

Termination: $ROO^* + ROO^* \longrightarrow$ inactive products

Antioxidant Defenses: α-Lipoic Acid and Dihydrolipoic Acid

As the earth began to evolve into an oxygen-rich environment, specific defenses to oxidation needed to occur. An antioxidant can be defined as any substance that, when present at low concentrations when compared to an oxidizable substrate significantly delays or prevents oxidation of that substrate (Halliwell, 1995). Within the body, antioxidants such as vitamin C, vitamin E, and glutathione protect tissues from oxidative damage. Oxidative stress occurs when the balance between oxidative and antioxidant processes favors oxidation.

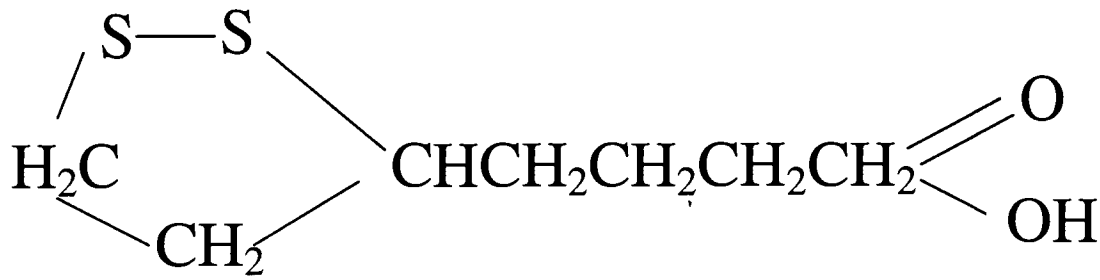
Thiols are ubiquitously distributed throughout aerobic life forms and also play a pivotal role in antioxidant defense in the system (Sen and Packer, 2000). The term thiol refers to a class of organic sulfur derivatives that are characterized by the presence of sulhydral residues (-SH) at their active site (Sen and Packer, 2000). Chemically, thiols are

classified as being mercaptans (C-SH). Most thiols contain a functional side chain CH_2-SH group of cysteine residues that serves as an active site for most thiols (Sen and Packer, 2000).

Most thiols have the ability to act as reducing agents. A reactive oxygen species has an ability to transfer electrons to other species. This process is known as oxidation. Thiols have a negative standard reduction potential and thus, act as electron acceptors. This accepting of the electrons by the thiol negates the effect of oxidation and protects the system from its effect. In an oxidant-thiol interaction, the oxidant is neutralized to a less toxic byproduct at the expense of the reducing power of the thiol, which in turn gets oxidized to a disulfide (C-S-S-C) (Sen and Packer, 2000). A thiyl radical (C-S*) is formed when a thiol (C-SH) loses its H atom from the -SH group or loses an electron from the sulfur, followed by a loss of a proton (Sen and Packer, 2000). At physiological pH, thiyl radicals are unstable and may recombine to form a disulfide (Wardman and von Sattag, 1995). In the body, specific reductases recycle disulfides back to thiols by using cellular-reducing equivalents such as NADH and its phosphoylated form, NADPH (Sen and Packer, 2000). By using this mechanism, cellular metabolism is coupled to maintain a favorable redox state for thiols.

The antioxidant α-lipoic acid is a thiol-based antioxidant. Lipoic acid is found in all kinds of prokaryotic and eukaryotic cells. Within the body, it serves as a cofactor in several 2-oxo acid dehydrogenases that take part in energy formation. Lipoic acid binds to acyl groups and transfers them from one part of the enzyme to another. The result of this occurring is the reduction of lipoic acid to dihydrolipoic acid (DHLA). DHLA can then be reoxidized back to lipoic acid by lipoamide dehydrogenase under the formation of NADH. Overall, lipoic acid and DHLA act as a redox couple that transfers electrons from the substrate lipoamide dehydrogenase to NAD^+.

Alpha Lipoic Acid



Dihydrolipoic Acid

Alpha-lipoic acid is a disulfide derivative of octanoic acid. Alpha lipoic acid contains a 1,2-dithiolane moiety, which contains a disulfide in a five-membered ring system. A chiral center is found at the three position on the 1,2 dithiolane ring. Alpha lipoic acid is also known as thioctic acid, 1,2-dithiolane-3-pentanoic acid, 1,2-thiolane-3-valeric acid, or 6,8-thioctic acid (Biewenga et. al, 1997).

The antioxidant activity of lipoic acid is mainly related to the chemical reactivity of its 1,2-dithiolane ring (Biewnga et al., 1997). The 1,2-dithiolane ring is more susceptible to reduction than other open-chain disulfides. The ring strain of lipoic acid provides its

physiological antioxidant capability, allowing it to scavenge free radicals (Biewenga et al., 1997).

Lipoic acid has been shown to be a very potent free radical scavenger. Lipoic acid has been found to scavenge hydroxy radicals (Scott et al., 1994), hypochlorous acid (Scott et al., 1991), and singlet oxygen (Devesagayam et al., 1993). However, it has been found that lipoic acid is not effective scavenging hydrogen peroxide (Scott et al., 1991) and the superoxide radical (Suzuki et al., 1991).

When considering the antioxidation effects of lipoic acid, its reduced form, DHLA must also be considered as lipoic acid is readily reduced to DHLA in the body. DHLA is thought to be more active than lipoic acid in the body, whereby its reactivity is caused mainly by its thiolate anions. DHLA is effective against scavenging hydroxy radicals (Suzuki et al., 1991), hypochlorous acid (Haenen et al., 1991) and the superoxide radical (Kagan et al., 1992). DHLA also exhibits direct scavenging activity that interacts with both water-soluble and lipid-soluble peroxyl radicals in the aqueous phase or in the hydrophobic domains of the lipid bilayer of liposomes or other membrane fractions (Kagan et al., 1992).

Lipoic acid binds to proteins and as a consequence, free lipoic acid has not been detected in human beings (Hermann et al., 1996). After therapeutic treatment, however, free lipoic acid can be observed in the blood plasma (Teichart et al., 1995). This free lipoic acid is determined as the amount of lipoic acid that is not bound to the amino acid lysine. In terms of biolavailability, lipoic acid supplementation from food in the natural diet is quite minimal. In order for a human to receive the previously administered dosage (Ziegler et al., 1995) of up to 1200 mg, a person would have to consume 1000 kg of pig heart, a tissue that has a relatively high level of lipoic acid. But upon therapeutic treatment, the amount of free

lipoic acid is relatively high. Upon administration, the concentration of lipoic acid can be determined in the blood plasma. Teichart and others (1995) found the concentration to be ~1.154 ng/mL of free lipoic acid in the blood plasma after application of 1 gram of lipoic acid to a healthy male human. The liver presumably eliminates lipoic acid and its absolute bioavialability was calculated between 20% and 38% (Hermann et al., 1996).

The fact that lipoic acid becomes reduced to form DHLA is very important when discussing the antioxidant properties of lipoic acid, as DHLA contributes a great deal to lipoic acid's antioxidant activity. A trait that thiols have is that they can reduce the disulfides of other thiols. In principle, this mechanism involves the thiol glutathione that becomes reduced to form GSH. GSH is then able to reduce the intermolecular disulfide of lipoic acid. However, this reaction proceeds too slowly to be recognized (Biewenga et al., 1997). A majority of the reduction from lipoic acid to DHLA occurs enzymatically. In the mitochondria, lipoamide dehydrogenase catalyzes the reduction of free lipoic acid by using NADH. In the cytosol, GSH catalyzes the reduction of free lipoic acid at the by using NADPH.

Within the body, α-lipoic acid links to a lysine group to form a linkage known as a lipoyl group. This lipoyl group is an important component of several multienzyme complexes. Such complexes that involve this α-lipoic acid-lysine linkage are the pyruvate dehydrogenase complex, the α-ketogluterate dehydrogenase complex, the brached-chain oxo-acid dehydrogenase, and the glycine cleavage system.

Lipoic acid is readily absorbed by the body after oral administration and is thus, highly bioavailable. Various studies (Harrison and McCormick, 1974; Podda et al., 1994) show that after either intraperitoneal or oral administration of lipoic acid, the antioxidant is

rapidly absorbed by the gut and taken up into various tissues. Here, the lipoic acid is metabolically reduced to DHLA. In a study done by Podda and others (1994), rats were administered α-lipoic acid for five weeks. At the end of the study, it was observed that various tissues readily absorbed α-lipoic acid, with the highest concentration being found in the heart. Control rats without lipoic acid supplementation displayed undetectable levels of lipoic acid in the liver, kidney, heart and skin (Podda et al., 1994).

Upon administration of α-lipoic acid, the maximal urinary excretion of lipoic acid occurs 3-6 hours after it is fed. When isotopically labeled lipoic acid is fed, approximately 45% of the lipoic acid is excreted in the urine during the first 24 hours and 3% of the original dosage is found in the feces, indicating that lipoic acid is readily absorbed by the gut (Harrison and McCormick, 1974). Four hours after administration of lipoic acid, the highest amount of radioactivity was observed in the liver, but radioactivity was observed in skeletal muscle after 24 hours.

Lipoic Acid/DHLA as an Antioxidant Recycler

Lipoic acid and DHLA appear to have the ability to regenerate other antioxidants such as vitamin E. Vitamin E is a major antioxidant that protects membranes from lipid peroxidation (Burton et al., 1981). It is thought that DHLA protects membranes from oxidation by recycling ascorbate, which in turn recycles vitamin E (Kagan et al, 1992). This is accomplished by interaction of DHLA with the NADH or NADPH electron transfer cycles to produce ascorbate. The ascorbate is then incorporated into vitamin E cycle to produce the active antioxidant tocopherol or vitamin E.

Effect of α-Lipoic Acid on Glycogen Storage

Lipoic acid is also believed to increase glycogen storage in cells by stimulating glucose transport in a similar fashion to that of the hormone insulin. Experiments done with regard to type I and type II diabetes have provided evidence that lipoic acid can facilitate this glucose transport. Jacob and others (1995) tested the effects of feeding lipoic acid to obese rats to determine its effect on glucose transport. Their results found that glycogen synthesis increased 38% with chronic administration of lipoic acid.

Estrada and others (1996) observed a dose dependent increase in glucose uptake in L6 muscle cells and adipocytes after treatment with lipoic acid. These cell lines were used as they are the only cell lines that express the GLUT-4 translocator. This GLUT-4 translocator is typically found in adult skeletal muscle and fat cells. The GLUT-1 (present in the same adult tissues but at lower levels) and GLUT-4 translocators move from the intracellular compartment to the cell membrane in response to insulin. Estrada and others (1996) observed that there was an increase in the translocation of GLUT-1 and GLUT-4 translocators intracellularly to the plasma membrane of the cell surface. The authors hypothesized that lipoic acid regulated the rate of glucose transport in these tissues by regulating phophatidylinositol 3-kinase. This kinase is involved in the recruitment of glucose transporters to the surface of the cell in response to elevated insulin levels. By increasing the translocation of these glucose transporters, lipoic acid may have an insulin-like effect, allowing more glucose to be taken up by the cells.

The Effect of α-Lipoic Acid as a Feed Supplement

The use of α-lipoic acid as a feed supplement has been studied by Berg and others (2001). This study consisted of a treatment group of twelve commercial hybrid barrows that were fed a short-term supplementation of 600 mg/pig/day of α-lipoic acid for five days prior to slaughter. This study observed that α-lipoic acid supplementation had a significant positive effect on loin 45 minute pH (6.48 vs. 6.04) and longissimus dorsi L* values (52.9 vs. 49.3). The L* values suggest that the pigs fed α-lipoic acid displayed a darker colored longissimus dorsi than the control group. Although the data was not significantly different, trends were observed showing that α-lipoic acid supplementation may positively affect drip loss in both the longissimus dorsi and semimembranosis muscles.

Effect of Oxidation on Muscle Proteins

Oxidation in meat can be very detrimental in a meat system from both a consumer and processor standpoint. The oxidation of meat can contribute to quality losses such as flavor deterioration, discoloration, destruction of nutrients, and the possible formation of toxic compounds (Kanner, 1994). Meat is susceptible to various forms of oxidation due to its high concentrations of unsaturated lipids, heme pigments, metal catalysts, and other various oxidizing agents that are present (Johns et al., 1989). The high protein concentration in meat is also susceptible to oxidation; leading to ROS modification of the protein via lipid oxidation, metal catalyzed oxidation or enzyme-catalyzed oxidation (Butterfield and Stadtman, 1997) postmortem. Proteins are especially susceptible to oxidation as the natural antioxidant defenses are slowly inactivated.

When proteins oxidize, the amino acids cysteine and methionine are thought to be the most susceptible amino acids to oxidation due to the presence of sulhydryl and sulfur in these amino acids. The subsequent alteration of amino acids attributed to oxidation does not require the presence of lipids. Metal-catalyzed protein oxidation that is site specific occurs widely and has been proposed to take place via hydroxyl free radicals (OH*) produced from H_2O_2 at specific iron binding sites on proteins (Stadtman and Oliver, 1991). These major modifications in the protein backbone can cause the protein to polymerize and possibly degrade.

The oxidation of proteins may cause the formation of protein aggregates. These aggregates that are formed via free radical chain reactions and are covalently bound. With the covalent nature of the bond, these protein aggregates are nondissociable in tissue. It is then possible that an amino acid side chain that is susceptible to free radical attack could produce a crosslink with an accessible amino acid from another protein (Xiong, 2000). This could occur by one of the following mechanisms (Xiong, 2000): 1) Oxidation of the cysteine SH groups to form disulfide links. 2) A complex formed between two oxidized tyrosine groups. 3) Interaction of an aldehyde group in one protein with the NH_2 group of a lysine residue in another protein. 4) A crosslink formed between two NH_2 groups of two lysine residues through a dialdehyde such as malonaldehyde. 5) A condensation reaction of protein free radicals.

In terms of functionality, myofibrillar proteins that are oxidized exhibit different levels of functionality when compared with their antioxidant-treated controls. These differences can be either beneficial or detrimental to the final product. This phenomenon can be attributed to the type, concentration, and length of oxidation on the protein. Total

inhibition of oxidation on a protein does not always improve the functionality of the product (Xiong, 2000). Antioxidants that facilitate protein-protein interaction usually improve the functionality of the meat. A distinct improvement in the gel-forming abilities of myofibrillar proteins was observed when lipid oxidation was completely inhibited (Wan et al., 1993; Xiong et al., 1993). However, it has also been shown that mild lipid oxidation of myofibrillar proteins can also aid in gel formation (Liu and Xiong 1996; Srinivasa and Xiong, 1996)

Oxidation has also been shown to play a detrimental role in calpain activity. Guttmann and others (1997) studied the affects of oxidation has on μ-calpain activity and autolysis at different calcium concentrations. This was accomplished by incubating μ-calpain with 100 μM hydrogen peroxide and measuring its activity using a fluorescent peptide as the substrate. The results showed that μ-calpain proteolysis of the fluorescent peptide were significantly decreased ($P < 0.05$) by the addition of 100 μM hydrogen peroxide and was reversible by the addition of dithioretinol (DTT). When calcium was present oxidation inhibited μ-calpain proteolysis of the substrates MAP-2 and tau. Immunoblots were then prepared to examine the effect of oxidation on autolysis of μ-calpain. These experiments displayed that the presence of an oxidant had no apparent effect on the extent of μ-calpain autolysis in either the 80 or 30 kDa subunits (Guttmann et al., 1997). However, the rate of autolysis was inhibited under these oxidative conditions. (Guttmann et al., 1997).

Oxidative stress on μ-calpain autolysis has been examined *in situ* as well. In research done by Guttmann and Johnson (1998) μ-calpain activity was stimulated in human neuroblastoma cells with proteolysis of the substrate being measured using a fluorescent

peptide. Calpain activity was stimulated through use of the calcium ionophore ionomycin, which increased the calcium concentration in the cell to 700-800 nM and maximum calcium concentration was achieved at 8-9 minutes of exposure to ionomycin. The effect of oxidative stress on calpain-mediated proteolysis was measured by the amount of proteolysis of MAP-2 and the microtubule associated protein tau, both highly sensitive calpain substrates. Oxidative stress was achieved by using doxorubicin or FF1, both known biological oxidants that can affect the cell. In both substrates, oxidation significantly lowered calpain activity and thus resulted in less calpain-mediated degradation of both substrates. Also, oxidation slowed the rate of autolysis but the extent of autolysis was unaffected by oxidation treatments (Guttman and Johnson, 1998). Furthermore, the addition of the antioxidant GSH prevented the oxidation effects on calpain activity (Guttman and Johnson 1998).

In agreement with these findings, a study by Rowe and others (2003) observed the oxidizing effects of irradiation on μ-calpain activity and autolysis. Western blot analysis and casein zymography revealed that irradiation slowed the rate of autolysis of μ-calpain and also suggested that μ-calpain was more active in non-irradiated samples, as observed by casein zymography. These casein blots revealed that oxidized beefsteaks that had been irradiated displayed minimal μ-calpain activity at 14 days post-irradiation (Rowe et al., 2003). This observation would suggest that μ-calpain was more active at earlier time points in non-oxidized steaks and had eventually decreased in its activity over time. This is in contrast to the irradiated samples that displayed minimal decrease in activity over the time points observed when measured by casein zymography. This suggests that μ-calpain may never have been active in these oxidized steaks but became active when introduced into the reduced environment, created by the addition of mercaptoethanol in the presence of the casein

zymography assay. These data supports the hypothesis of inactivation of μ-calpain by oxidation can be reversed when introduced into a reducing environment (Rowe et al., 2003).

Oxidation can also have detrimental affects on the proteins that are involved in Ca^{2+} regulation of the muscle cell. In a study conducted by Klebl and others (1998), oxidized rabbit muscle exhibited a reduction in SERCA activity by 45-50% in four hour stimulated muscle. This reduced activity of the SERCA precedes the isoform switch of fast-twitch SERCA1a to the slow-twitch SERCA2a with prolonged electrical stimulation (Hicks et al., 1997). An in vitro study on isolated sarcoplasmic reticulum suggest that a hydroxy radical (Xu et al., 1997) may be a culprit for inducing decreased activity by direct attack of the ATP binding site on the SERCA. This was accomplished by incubating purified SERCA with a hydroxyl radical-generating system and also ATP to protect the ATP binding site. An activity assay was done to determine the activity of the SERCA. Their results show that incubation with ATP protected the binding site and a significant reduction in activity was not observed.

Further evidence to show the effect of oxidation on SERCA function was reported by Grover and other (2003). This study observed the effects of peroxynitrite on SERCA-2b in porcine coronary artery smooth muscle. The use of peroxynitrite was used because it being produced during such disorders as cell injury, atherogenic inflammatory response, and ischemia-reperfusion. Peroxynitrite is gernerated from nitric oxide and superoxide. This study observed that peroxynitrite and H_2O_2 treatments inhibited Ca^{2+} uptake by the cells. It was also observed that oxidation by both treatments produced oligomers that reacted with the SERCA-2b antibody used in western blot detection. These oligomers migrated at approximately 260 kDa, which is higher than the intact 110 kDa SERCA. The authors

suggested that the SERCA-2b in the coronary artery might be part of a much larger oligomeric complex than what is observed in skeletal or cardiac muscle.

The ryanodine receptor has been shown to be susceptible to oxidation as well. A study done by Donoso and others (2000) showed that oxidation of the sulfhydryl groups of the ryanodine receptor overrides the inhibition effects of Mg^{2+} and causes a loss of control of the protein, resulting in a larger release of Ca^{2+}. Oxidation of the rabbit muscle was accomplished by incubating the samples with thimerosal for various lengths of time. Mg^{2+} may be a potent inhibitor of the ryanodine receptor because it competes with the Ca^{2+} at the activation site (Copello et al., 2002) or at the inhibition site (Copello et al., 2002), thus canceling out the effects that Ca^{2+} binding would have for activation or inhibition. Also, Mg^{2+} may bind with ATP to reduce the amount of free ATP able to bind to the ryanodine receptor for activation (Sonnleitner et al., 1997).

Several other studies have been conducted to display the ryanodine receptor's susceptibility to oxidation. Site selective oxidation of the hyperreactive thiols on the ryanodine receptor has been shown to be susceptible to oxidation by quiones such as doxorubicin (Feng et al., 1998). This same study also observed that oxidation of these thiols caused an increase in channel activation (Feng et al., 1998). This finding agrees with the findings of Zable and others (1997) that observed that DTT and other thiol reductants such as glutathione (GSH) and mercaptoethanol inhibited ryanodine receptor activity and oxidized glutathione increased ryanodine receptor activity. However, a study done by Heunks and others (2001) used C_2C_{12} myoblasts and real-time confocal imaging of Ca^{2+} concentration (measured in fluorescence) to determine the effect of the oxidant nitric oxide on Ca^{2+} release. It was observed that nitric oxide effectively decreased the concentration of intracellular Ca^{2+}

concentration in the myotubes at concentrations of 10 μM and 50 μM nitric oxide. This effect was partly reversible by addition of DTT, a thiol reductant (Heunks et al., 2001). It was interesting to note that the addition of 100 μM nitric oxide into the system with DTT was not significantly different from the control, but this may be attributed to small sample size (n = 9) for this treatment, as opposed to n = 15 for the other treatments (Huenks et al., 2001). The authors suggested that a thiol modification of ryanodine receptor by nitric oxide was the cause of the decreased levels of intracellular Ca^{2+} (Heunks et al., 2001) and that nitric oxide has a very complex effect on the ryanodine receptor and on Ca^{2+} regulation.

Oxidation of sulfhydryl groups on the cytoplasmic side of the ryanodine receptor can enhance the effect of modulators such as adenine dinucleotide and caffeine, both activators of the ryanodine receptor (Oba et al., 2002). This suggests that the magnitude of the channel response may be regulated by the redox status of the ryanodine receptor (Oba et al., 2002).

Another modulator of the ryanodine receptor is calmodulin, which both inhibits (low Ca^{2+}) and promotes ryanodine receptor activity (high Ca^{2+}). Balog and others (In Press) suggested that oxidation of methionine residues on calmodulin could potentially lead to altered regulation of the ryanodine receptor during stress of the animal. By using site-directed mutageneis of methionine residues on calmodulin, oxidation of all nine methionines abolished functional interaction of calmodulin to the ryanodine receptor (Balog et al., In Press). The authors suggested that this oxidation of calmodulin might contribute to the dysfunction of the ryanodine receptor during oxidative stress by altering the binding capabilities of calmodulin to the ryanodine receptor (Balog et al., In Press).

Summary

This literature review addressed the topics of glycogen storage, anaerobic metabolism of glucose, Ca^{2+} regulation during muscle contraction and relaxation, proteolysis by μ-calpain, factors that influence water-holding capacity and tenderness, and effect of oxidation on meat quality. The interpretations of numerous studies have been compiled to show relationships between postmortem factors such as pH decline, proteolysis, and oxidation with meat quality attributes such as water-holding capacity and tenderness. A novel way of preventing oxidation in muscle in early times postmortem may be to supplement the finishing ration with lipoic acid, a potent free radical scavenger. Beyond the antioxidant qualities of lipoic acid, other added benefits of lipoic acid supplementation have been observed as well, such as the effectiveness of its reduced form DHLA as a free radical scavenger, its ability to recycle other antioxidants such as vitamin E, and its ability to promote glycogen storage as macroglycogen instead of proglycogen, which may aid in the rate of pH decline. Collectively, this literature review provides supporting evidence towards the hypothesis that lipoic acid supplementation may decrease negative effects of oxidation on proteins involved in Ca^{2+} regulation and proteolysis, which may in turn enhance the quality of pork being produced.

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RELATIONSHIPS BETWEEN OXIDATION AND μ-CALPAIN AUTOLYSIS, DEGRADATION OF CA^{2+}-REGULATING PROTEINS, AND PORK QUALITY

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Abstract

The objective of this study was to determine the effects that oxidation may have on pork quality attributes and protein degradation. Fifty-four commercial hybrid pigs of similar genetics were harvested at a commercial facility at a final live weight of approximately 118 kg. Measurements for longissimus dorsi (LD) pH were taken at 1 h and 24 h postmortem. LD samples were then collected at 1 h, 24 h, 96 h, and 7 d postmortem for western blot analysis. Samples from the LD were also collected for glycogen (1 h and 24 h), oxidation (24 h), drip loss (2 d), and Warner-Bratzler Shear (WBS) at 2 d and 21 d postmortem. Correlations between quality attributes revealed that increased lipid oxidation was correlated ($P < 0.05$) to decreased pH. An increase in lipid oxidation was also correlated ($P < 0.05$) to decreased WBS. Furthermore, increased drip loss was correlated ($P < 0.05$) to decreased 24 h pH. Western blot analysis to determine the extent of autolysis of the proteolytic enzyme μ-calpain showed an increase in the extent of autolysis was correlated ($P < 0.05$) to decreased 1 h pH and lower WBS. An increase in the degradation of the Ca^{2+}-regulating protein

sarcoplasmic reticulum Ca^{2+}-ATPase pump-1 (SERCA-1) revealed correlations ($P < 0.05$) with increased lipid oxidation. Increased degradation of another Ca^{2+} regulating protein, the ryanodine receptor (RYR1), was correlated ($P < 0.05$) to higher pH at 24 h. Increased degradation of the SERCA-1, RYR1, and desmin was correlated ($P < 0.05$) to further μ-calpain autolysis and lower WBS. The correlations suggest that the extent of proteolysis by μ-calpain may be affected by factors that control Ca^{2+} regulation and that Ca^{2+} regulation in the cell may be affected by proteolysis. This proteolysis could then affect quality attributes such as tenderness.

Introduction

Many biochemical factors present in early postmortem muscle tissue can influence pork quality. Some of these factors include pH of the muscle and oxidative environment in the muscle cell. It is well documented that pork products with a fast pH decline and/or low ultimate pH can have detrimental quality attributes such as high drip loss (Offer and Knight, 1988; Lonergan, Huff-Lonergan, Rowe, Kuhlers, & Jungst, 2001). As the rate of pH decline increases, the amount of tissue oxidation also increases due to an increase in metabolism (Nurnberg et al., 2002). Pre-slaughter stress has also been associated with lower ultimate pH and increased oxidation (Juncher et al., 2000). An oxidative environment has also been shown to decrease the activity of the enzyme μ-calpain (Guttman, Elce, Bell, Isbell, & Johnson, 1997; Guttman and Johnson, 1998; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2003).

μ-Calpain is a ubiquitous Ca^{2+}-dependant protease responsible for degrading cytoskeletal and myofibrillar proteins (for review, see Goll, Thompson, Taylor, Ouali, &

Chou, 1999) that may influence pork quality. A loss of control of Ca^{2+}-regulating proteins such as the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) or the ryanodine receptor could result in an increase in the concentration of Ca^{2+} in the sarcoplasm. This increase in Ca^{2+} can affect early postmortem environmental factors such as pH by causing an increase in metabolism and thus a faster pH decline (Kuchenmesiter et al., 1999a; Kuchenmeister, Kuhn, Wegner, Nurnberg, & Ender, 1999b). This increase in Ca^{2+} concentration could be detrimental to pork quality by possibly inducing PSE conditions. However, a moderate increase in sarcoplasmic Ca^{2+} may also aid in the activation of μ-calpain and allow for the degradation of cytoskeletal proteins that may influence pork quality attributes.

Upon activation of μ-calpain, proteolysis of cytoskeletal proteins by μ-calpain can have a positive affect on both tenderness and water-holding capacity. The cytoskeletal protein desmin has been shown to be susceptible to proteolysis by μ-calpain (O'Shea, Robson, Huiatt, Hartzer, & Stromer, 1979; Huff-Lonergan et al., 1996). The increased degradation of desmin has been related to increased tenderness (Koohmaraie, Kennick, Elgasim, & Anglemier, 1984; Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson, & Robson, 1996; Melody, Lonergan, Rowe, Huiatt, Mayes, & Huff-Lonergan, 2003) and increased water-holding capacity (Rowe, Huff-Lonergan, & Lonergan, 2001a; Melody et al., 2003).

Several Ca^{2+} regulating proteins are susceptible to oxidation and to proteolysis by μ-calpain. The SERCA is a membranous protein that functions to remove Ca^{2+} from the sarcoplasm upon a signal to begin relaxation of the muscle. The SERCA pump can be found in three different isoforms, encoded by a highly conserved family of genes (Loukianov, et al., 1998). In adult tissue, SERCA-1a proteins are exclusively expressed in fast-twitch skeletal

muscle (Brandl, Green, Korczak, & MacLennan, 1987). The SERCA-2 gene encodes for the SERCA-2a and SERCA-2b isoforms. SERCA-2a is found exclusively in cardiac, slow-twitch skeletal muscle, and smooth muscle (Brandl et al., 1987). SERCA-2b is expressed in most cell types but found in high levels in smooth muscle (Lytton, Zarain-Herzberg, Periasamy, & MacLannan, 1989). SERCA-3a and SERCA-3b are expressed in cell types such as platelets, lymphocytes, endothelial cells, and epithelial cells (Wuytack, Dode, Baba-Aissa, & Raeymaekers, 1995). Increased oxidation of the SERCA pump has been shown to detrimentally influence the function of SERCA (Klebl, Ayoub, & Pette, 1988; Hicks, Ohlendieck, Gopel, & Pette, 1997; Xu, Zweirer, & Becker, 1997). The SERCA can be degraded by μ-calpain as well (Wang, Roufogalis, and Villalobo, 1988a and 1988b; Seidler, and Cucchetti, 1998). Degradation of the SERCA could potentially affect the function of the SERCA in postmortem muscle.

Another protein regulating Ca^{2+} concentration in the muscle cell is the ryanodine receptor. The ryanodine receptor functions to release Ca^{2+} from the lateral cisternae of sarcoplasmic reticulum into the sarcoplasm. In mammals, there are three different isoforms of the ryanodine receptor: RYR1, RYR2, and RYR3 (for review, see Fill and Copello, 2002). The predominant isoform found in skeletal muscle is the RYR1 isoform. RYR2 is abundantly found in cardiac muscle. RYR3 is found in striated muscles but at relatively low levels. Increased oxidation has been shown to override the natural inhibition of Mg^{2+} (Donoso, Aracena, & Hidalgo, 2000) and cause a loss of control of the ryanodine receptor (Feng, Liu, Abramson, & Pessah, 1998; Zable, Favero, & Abramson, 1997), resulting in a large amount of Ca^{2+} being released into the sarcoplasm. The ryanodine receptor can also be degraded by calpains (Brandt, Caswell, Brandt, Brew, & Mellgren, 1992). Degradation of

the ryanodine receptor by m-calpain can cause a loss of inhibition, resulting in an increase of Ca^{2+} to be released into the sarcoplasm (Iino, Takano-Ohmuro, Kawana, & Endo, 1992).

The extent of oxidation, rate of pH decline, ultimate pH, and protein degradation are important factors contributing to pork quality attributes such as drip loss and tenderness. Therefore, the objective of this study was to study the relationships between these factors and pork quality attributes. Furthermore, this study examined the relationships between μ-calpain autolysis and the degradation of Ca^{2+}-regulating proteins (SERCA and ryanodine receptor) and desmin.

Materials and Methods

Fifty-four commercial hybrid pigs (26 barrows and 28 gilts) developed for adequate muscle mass and enhanced quality (Triumph TR4 sire x PIC C22 dams) from Triumph Pork Group LLC were used in this study. These pigs were randomly separated into three treatment groups: a control (traditional finishing diet) diet was fed to 19 pigs, a low additive (control + 8 mg lipoic acid/kg live body weight per pig) diet was fed to 16 pigs, and high additive (control + 16 mg lipoic acid/kg live body weight per pig) diet was fed to 19 pigs, with supplementation beginning at approximately 95 kg. All pigs were fed their respective diets until reaching their finished weight of ~118 kg. These pigs were fed at the New Ulm experiment station under the supervision of Triumph Pork Group LLC personnel in New Ulm, Minnesota.

The pigs were humanely slaughtered using standard commercial practices over a two-day period at the Farmland Slaughter Facility in Crete, Nebraska. Pigs from each treatment group were represented on each harvest day. Both harvest groups were held off feed overnight (8 h) prior to harvest. Measurements for pH (Mettler-Toledo glass-tipped probe, Mettler-Toledo Process Analytical Inc., Wilmington, MA) were taken at 1 h and 24 h postmortem in the longissimus dorsi (LD) on the right side of the carcass. At 1 h and 24 h postmortem, samples (approximately 20 g) were taken from the LD anterior to the tenth rib on the left side of the carcass. All samples removed at or before 24 h postmortem were flash frozen in liquid nitrogen, sealed in WhirlPak bags (Nasco Sampling Products, Fort Atkinson, WI), stored on dry ice, and transported to Iowa State University, where they were stored at -80°C. Upon fabrication into primal cuts, loins from the right side of the carcass were wrapped and taken to the University of Missouri Meat Laboratory where they were aged at 4°C upon analysis for Warner-Bratzler shear force (WBS) and drip loss.

Drip loss was determined from the LD samples and calculated from the 24 h weight loss in the shelf life display at 2 d postmortem. One-inch thick chops were removed anterior to the tenth rib, weighed, placed on a Styrofoam retail tray, over wrapped with O_2 permeable film. After 24 h of storage at 2°C, the chops were reweighed and moisture loss was calculated as a percentage of the original weight.

WBS measurements were also taken from the LD. One-inch thick chops were removed from the loin at 2 d postmortem. Chops were cooked in a MagiKitch'n (Blodgett Co., Quakertown, PA) belt grill conveyer cooker at 2 d postmortem and 21 d postmortem. Raw and cooked weights were recorded, and temperature was taken using a HH-21 calibrated thermometer (Omega Engineering, Inc., Stanford, CT) as the chops exited the MagiKitch'n

belt grill conveyer cooker. The top, bottom, and preheat temperature of the belt grill was set to 177°C with a constant cook time of 4 min. The distance between heating platens was 2.16 cm. After reaching their endpoint temperatures cooked chops were placed on a large tray and covered with an oxygen-permeable clear plastic wrap. The chops were cooled overnight at approximately 4°C. The following day, six core samples (1.27 cm in diameter) were excised from each loin chop parallel to the muscle fiber, and sheared perpendicular to the fiber orientation using the standard Warner-Bratzler shear (G-R Manufacturing, Manhattan, KS).

At 96 h and 7 d postmortem approximately 20g of uncooked tissue were taken from each chop anterior to the tenth rib, frozen at -80°C, and transported on dry ice to Iowa State University for further analysis.

Glycogen Concentration Analysis

Samples for glycogen concentration were taken from the left side of the animal from the LD at 24 h post mortem. Samples were collected, flash frozen in liquid nitrogen, and then stored at -80°C until analysis. Concentration of glycogen was determined using the methods of Bergmeyer (1983). Samples of 0.5 g of muscle were homogenized using a motor-driven 30 mL Potter-Elvehjem tissue grinder with a PTFE pestle (Wheaton Science, Millville, NJ) in 2.5 mL of 0.6 N perchloric acid. The homogenate (200 μL) was neutralized with 100 μL of 1 M potassium carbonate and incubated with 2.0 mL of amyloglucosidase (Sigma Chemical Co., St. Louis, MO) in 0.2 M acetate buffer (pH 4.8) for 120 min at 40°C in a Fisher Isotemp Water Bath (Indiana, PA). The amyloglucosidase was precipitated by the addition of 0.6 N perchloric acid. The hydrolyzed sample was centrifuged at 1500 x g for 15 min at 4°C. Total glucosyl units (μM glucosyl units/gram of tissue) of the supernatant were

determined using premixed reagents (Glucose HK Assay Reagent, Sigma Chemical, Co., St. Louis, MO). After 15 min of incubation at room temperature, the absorbance was measured at 340 nm (Ultraspec 3000 UV/Visible Spectrophotometer, Amersham Pharmacia Biotech, Piscataway, NJ). μM Free glucose was calculated by using the following calculation:

$[(OD_{340}$ sample) – (OD_{340} blank)/(OD_{340} reference standard) – (OD_{340} blank)] x 8.88.

μM Total glucosyl units was calculated by the following calculation:

$[(OD_{340}$ sample) – (OD_{340} blank)] x 291.2

The μM total glucosyl units from glycogen was then calculated as:

[Total glucosyl units – Free Glucose].

Lipid Peroxidation Analysis

Lipid peroxidation samples were taken from the left side of the animal from the LD at 24 h post mortem. Samples were collected, immediately flash frozen in liquid nitrogen, and then stored at -80°C until analysis. Lipid peroxidation was determined by using a Calbiochem Lipid Peroxidation Assay Kit (Cat. No. 437634, San Diego, CA). This kit takes advantage of a chromogenic reagent, 10.3 mM N-methyl-2-phenylindole in acetontirile (R1), which reacts with malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (HNE) at 45°C. Thus, a color change will accompany the presence of these end products that result from the peroxidation of fatty acids and related esters. This absorbance of color change can be observed at a maximal absorbance of A_{586}.

Samples of 0.5 g of muscle were homogenized using a motor-driven 30 mL Potter-Elvehjem tissue grinder with a PTFE pestle (Wheaton Science, Millville, NJ) in 5 mL 20mM

Tris-HCl (pH 7.4) and 200 µL of 1M butylated hydoxy tolulene (BHT) in ethanol to prevent further sample oxidation. The homogenates were centrifuged at 3000 x g for 10 min at 4°C. 200 mL of the supernatant was collected for analysis. Standard curves were used to determine the concentrations of malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (HNE) in the muscle sample. Standard curves were prepared fresh using 10 mM 4-HNE in acetontrile and 10mM MDA in 20 mM Tris-HCl (pH 7.4) to result in a 100-fold (v/v) dilution. These dilutions were then added to 650 µl of premixed R1 that was diluted in 100% methanol (6 mL of 100% methanol:18mL of R1) in a clean glass test tube to be used to make the standard curve. In order to make the standard curve, the MDA and HNE were diluted in 20 mM Tris-HCl (pH 7.4) to make final concentrations of 0 µM, 1 µM, 3 µM, and 5 µM MDA or HNE for the standard curve. In a clean glass test tube, 650 µl of premixed R1 that was diluted in 100% methanol (6 mL of 100% methanol:18mL of R1) was added to 200 µl of the sample supernatant and vortexed for 3-4 seconds. A sample blank was also made using 200 µl of 20mM Tris-HCl instead of 200 µl of supernatant. Immediately, 150 µl of the premixed 15.4 M methanesulfonic acid (R2) was added to the sample, vortexed, and sealed with a plastic stopper. The samples and standard curves were then incubated at 45°C for 40 min in a Fisher Isotemp Water Bath (Indiana, PA). After the samples and standard curves were cooled in ice, absorbance was determined at 586 nm (Ultraspec 3000 UV/Visible Spectrophotometer, Amersham Pharmacia Biotech, Piscataway, NJ). Calculation of the concentration of MDA + HNE was determined by the following equation: [MDA + HNE]=(A_{sample} -A_{blank}) x 5/(slope of standard curve of either MDA or HNE).

SDS-PAGE Sample Preparation

Samples (approximately 20 g) were taken at 1 h, 24 h, 96 h and 7 d for the LD at the portion anterior to the tenth rib. Whole muscle samples were prepared using the methods described by Lonergan, Huff-Lonergan, Rowe, Kuhlers, and Jungst (2001). Five mL of a buffer containing 10 mM sodium phosphate and 2% SDS (pH 7.0) was added to 0.2 g of each sample. The sample was homogenized using a motor-driven 30 mL Potter-Elvehjem tissue grinder with a PTFE pestle (Wheaton Science, Millville, NJ). Samples were centrifuged at 1500 x g (Sorvall Legend RT Centrifuge, Sorvall Products, L.P., Newton, CT) for 15 min at 20°C. The concentration of protein in the sample was determined using methods described by Lowry et al. (1951) for each sample using prepared reagents (DC Protein Assay, Bio-Rad, Hercules, CA). Protein concentration was then adjusted to a concentration of 6.4 mg/mL by diluting with deionized water. One volume of each diluted sample was combined with 0.5 volumes of tracking dye solution (3 mM EDTA, 3%[wt/vol] SDS, 30%[vol/vol] glycerol, 0.001% [wt/vol] pyronin Y, and 30 mM Tris-HCl, pH 8.0) and 0.1 volume of ß-mercaptoethanol for a final protein concentration of 4 mg/mL. The gel samples were heated for 20 min at 50°C and frozen at -80°C for storage. Whole muscle samples were used to analyze μ-calpain, SERCA-1, RYR1, and desmin using SDS-PAGE and western blotting techniques.

SDS-PAGE Gel System

The polyacrylamide separating gels were prepared using a solution containing acrylamide:N,N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] sodium dodecyl sulfate (SDS), 0.05% [vol/vol] tetramethylethylenediamine (TEMED), 0.05% [wt/vol]

ammonium persulfate, and 0.375 M Tris-HCl, pH 8.8 as described by Lonergan et al. (2001). A 10% polyacrylamide separating gel was used for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis of desmin. A 9% polyacrylamide separating gel was used for SDS-PAGE analysis of μ-calpain. An 8% polyacrylamide separating gel was used for SDS-PAGE analysis of the SERCA-1. A 6% polyacrylamide separating gel was used for SDS-PAGE analysis of the RYR1. A 5% gel (acrylamide:N,N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.125% [vol/vol] TEMED, 0.075% [wt/vol] ammonium persulfate, and 0.125 M Tris-HCl, pH 6.8) was used for a stacking gel on the 8%, 9%, and 10 % polyacrylamide separating gels. A 4% stacking gel of same composition was used for the 6% polyacrylamide gel.

Samples for μ-calpain and the RYR1 were run on SE 280 Hoefer Tall Mighty Small (Hoefer Scientific Instruments, San Francisco, CA) electrophoresis units. Gels (10 cm wide x 10 cm tall x 1.5 mm thick) for SERCA-1 and desmin were run on an SE 260 Hoefer Mighty Small II electrophoresis unit. The running buffer contained 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 0.1% (wt/vol) SDS. Gels were loaded with 100 μg of total protein for μ-calpain, 60 μg of total protein for SERCA-1, 160 μg of total protein for RYR1, and 30 μg of total protein for desmin. Gels loaded for μ-calpain analysis were run at a constant voltage (Power Pac 1000, Biorad, Hercules, CA) of 120 V for approximately 4 h. Gels for SERCA-1 analysis were run at a constant voltage (Electrophoresis Power Supply EPS 300, Pharmacia Biotech, Piscataway, NJ) of 20 V for approximately 16 h. Gels loaded for RYR analysis were run at constant amperage (Power Pac 1000, Biorad, Hercules, CA) of 3 mA per gel for approximately 24 h. Gels loaded for desmin were run at constant voltage (Electrophoresis Power Supply EPS 300, Pharmacia Biotech, Piscataway, NJ) at 120 V.

After electrophoresis, gels for μ-calpain, SERCA-1, and desmin were transferred to polyvinylidene difluoride (PolyScreen, NEN Life Science Products, Boston MA) transfer membranes using a TE22 Mighty Small Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) at a constant voltage (FB570, Fisher Scientific, Pittsbug, PA) of 90 V for 1.5 h. The transfer buffer consisted of 25 mM Tris, 192 mM glycine, and 15% [vol/vol] methanol. The temperature of the transfer buffer was maintained using a refrigerated circulating water bath (Ecoline RE106, Lauda Brinkman, Westbury, NY) set at 0.4°C. Gels for the RYR1 were transferred to PVDF (PolyScreen, NEN Life Science Products, Boston MA) membranes using a TE 62 Transfer Cooled Unit (Hoefer Scientific Instruments, San Francisco, CA) at constant amperage of 400 mA for 2 h and 1000 mA for 3 h. The same transfer buffer was used with exception to 10 % [vol/vol] methanol being used. The temperature of the transfer buffer was maintained between 4°C and 8°C by using a refrigerated circulating water bath (Ecoline RE106, Lauda Brinkman, Westbury, NY) set at –5°C for the first 2.5 h and –10°C for the remaining 2.5 h of the transfer.

Following transfer, the membranes were first incubated in a 20 mL blocking solution containing PBS-Tween (80 mM disodium hydrogen orthophosphate, anhydrous, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, 0.1% polyoxyethylene sorbitan monolaurate [Tween 20]) and 5% [wt/vol] non-fat dry milk for one h. A primary antibody was then used for each of the desired proteins. These primary antibodies included monoclonal (mouse) anti-μ-calpain (clone # 9A4H8D3, Affinity BioReagents, Golden, CO) for a minimum of 12 h (4°C) at a 1:10,000 dilution with PBS-Tween, monoclonal (mouse) anti-SERCA-1 ATPase (Clone # VE12$_1$G9, Affinity BioReagents, Golden, CO) for 2 h (room temperature) at a dilution of 1:60,000 with PBS-Tween, monoclonal (mouse) anti-

RYR1 antibody (Clone # 34-C, Affinity BioReagents, Golden, CO) for a minimum of 12 h (4°C) at a 1:5000 dilution with PBS-Tween, and polyclonal rabbit anti-desmin (Cat # V2022 Biomeda, Foster City, CA) for 2 h (room temperature) at a 1:10,000 dilution with PBS-Tween. Following incubation in the primary antibody, the membranes were washed three times (10 min each) with PBS-Tween. Secondary antibodies were then applied. Secondary antibodies included goat-anti-mouse-horse radish peroxidase (HRP) (Cat # A2554, Sigma, St. Louis, MO) in a 1:5000 dilution (μ-calpain, RYR1) or a 1:10,000 dilution (SERCA-1) with PBS-Tween for 1 h (μ-calpain, SERCA-1) or 2 h (RYR1); goat-anti-rabbit-HRP (Cat # A9169 Sigma, St. Louis, MO) in a 1:5000 dilution with PBS-Tween for 1.5 h (desmin). After incubation in the secondary antibody, the membranes were washed three times (10 min each). Chemiluminescence (ECL+Plus, Amersham Pharmacia Biotech, Piscataway, NJ) was used according to kit instructions. To detect horseradish peroxidase tagged primary anitbodies, a 16-bit megapixel CCD camera FluorChem 8800 (Alpha Innotech, San Leandro, CA) and FluorChem IS-8800 software (Alpha Innotech, Sanleandro, CA) were used.

Densitometric measurements of the intact protein and degradation products of SERCA-1, RYR1, and desmin were determined using FlourChem IS 8800 software (Alpha Innotech, San Leandro, CA). Analysis of the relative intensities of the 80 kDa, 78 kDa, and 76 kDa forms of the intact or autolyzed catalytic subunits of μ-calpain was determined using the autogrid analysis tool. Each band was reported as a percentage of the total intensity of the 3 bands measured within each lane.

The amount of SERCA-1 used in analysis was determined by calculating percentage of the 7 d SERCA-1 degradation products, A and B, when compared to the degradation A and B from a standard run on every gel. The standard was a porcine LD sample (Pig # 3876)

at 7 d postmortem that showed both degradation A and B. The autogrid analysis tool was used to measure the intensity of SERCA-1 degradation bands. This analysis is reported as the sample percentage of the standard percentage of each band.

The RYR1 was analyzed by measuring the area and intensity of a lower molecular weight band identified as band B on 24 h samples and a higher molecular weight band identified as band A on 96-h samples by using the spot-denso analysis tool from the FlourChem IS 8800 software (Alpha Innotech, San Leandro, CA). This area was then compared to a standard run on every gel. Band B at 24 h was compared to the band B of a porcine LD sample (Pig # 3801) at 24 h postmortem. Band A at 96 h was compared to the band A of a porcine LD sample (Pig # 3891) at 96 h postmortem. The results used in this analysis are reported as a percent of the standard.

The analysis of desmin was determined by measuring the area of the intact 55 kDa band and comparing it to a control sample run on every gel. The spot-denso analysis tool from the FlourChem IS 8800 software (Alpha Innotech, San Leandro, CA) was used to measure the area and intensity of desmin. The amount of intact desmin was reported as a percentage of the intact desmin from a control sample. The control sample used was a porcine LD sample at 1 h postmortem that had both the intact and degradation product present. Analysis of the desmin degradation A was done in a similar fashion as the area and intensity of the degradation product was compared back to the degradation product of the same control sample as described for the intact desmin. The amount of desmin degradation product is reported as a percentage of the control sample

Statistical Analysis

Data were analyzed using PROC GLM and PROC CORR of SAS (version 8.2, SAS Institute. Cary, NC). Each of the pigs (n = 54) served as replications in the experiment. At each time point, comparisons were made between the three diets (control, low dosage, and high dosage). Due to no significant effect of lipoic acid treatment on the parameters analyzed in this study, the data was then pooled for further analysis.

Results and Discussion

Effect of Lipoic Acid

There was no significant ($P > 0.05$) effect of lipoic acid treatment on any of the factors measured in the LD (1 h pH, 24 h pH, total glycogen at 1 h and 24 h, 4-Hydroxy Non-Enal concentration at 24 h (HNE), malondialdehyde (MDA) concentration at 24 h, Warner-Bratzler shear (WBS) force at 2 d postmortem storage or 22 d postmortem storage). However, the dosage and the manner of administration of the treatment were not sufficient in order to determine lipoic acid's effect on pork quality. The data from the biochemical attributes and quality attributes of the LD are summarized in Table 1.

There was variation in the amount of LD lipid oxidation at 24 h among the pigs (Table 1). These pigs were of similar genetics and also were fed a similar diet. Based on these differences in oxidation, our objective was to determine relationships between extent of lipid oxidation and pork quality attributes such as 1 h pH, 24 h pH, 2 d drip loss, total glycogen at 1 h and 24 h, and WBS at 2 d and 22d. Furthermore, we studied the effects of μ-calpain autolysis and the subsequent degradation of Ca^{2+}-regulating proteins and desmin to determine their relationships with pork quality attributes.

Correlations Between Quality Attributes

The correlations between the quality attributes are summarized in Table 2.

An analysis of the quality attributes (1 h pH, 24 h pH, HNE concentration at 24 h, MDA concentration at 24 h, and WBS at 2 d and 22 d) revealed no significant correlations ($P > 0.05$) with the concentration of glycogen in the LD. The lack of a significant correlation between the concentration of glycogen at 1 and 24 h postmortem with pH measured at 1 h and 24 h postmortem is in agreement with a study by Lonergan et al. (2001). Lonergan et al. (2001) observed differences ($P < 0.01$) in the mean values for pH at 45 minutes postmortem between a line selected for lean growth efficiency and a control line (45 min pH = 6.09 in the lean growth line versus 6.35 in the control line). However, Lonergan et al. (2001) observed no differences ($P > 0.05$) in the concentration of glycogen between the two lines at 15 minutes postmortem (29.49 in the lean growth line versus 31.60 in the control line).

Oxidation in meat systems can play a detrimental role in the quality and functionality of meat (Wan et al., 1993; Xiong et al., 1993). A negative ($P < 0.05$) correlation existed between the concentration of HNE and WBS at 2 d postmortem ($r = -0.291$), suggesting an increase in oxidation was related to a decrease in WBS. A similar correlation existed when the concentration of MDA was compared to WBS at 2 d postmortem ($r = -0.261$). However, this correlation was not significant at the $P < 0.05$ level ($P = 0.056$). This could be a result of a decrease in the function of the SERCA as oxidation could alter ATP binding sites on the SERCA, thus decreasing the function of a membranous protein such as the SERCA pump (Xu et al., 1997). Oxidation could then possibly hinder the function of the SERCA function, which may promote increased meat tenderness by decreasing the amount of Ca^{2+} being

removed from the sarcoplasm. This could then promote the activity of the Ca^{2+}-dependant protease, μ-calpain.

Analysis of the extent of lipid oxidation in this study showed that 24 h pH was negatively ($P < 0.05$) correlated to both lipid oxidation measurements of HNE ($r = -0.349$) and MDA ($r = -0.367$). These correlations suggest that samples with low pH at 24 h may also have more lipid oxidation. This correlation supports the findings of Nurnberg et al. (2002). Their study observed higher lipid oxidation in malignant hyperthermia-susceptible pigs (1.33 nmol/g wet weight) than in malignant hyperthermia-resistant pigs (0.81 nmol/g wet weight). Although pH was not recorded in this study by Nurnberg et al. (2002), malignant hyperthermia-susceptible pigs typically have a faster rate of pH decline than MHR pigs (Kuchenmesiter et al., 1999a; Kuchenmeister et al., 1999b). Another study by Juncher et al. (2000) studied the effects of pre-harvest stress on oxidative stability of the meat. This study found that loins from carcasses that had a lower ultimate pH (pH = 5.7 vs. 5.9 and 6.2) also had a significant ($P < 0.001$) increase in lipid oxidation. Juncher et al. (2000) suggested that the autoxidation of oxymyoglobin is responsible for the initiation of lipid oxidation. The autoxidation of oxymyoglobin is acid-catalyzed, thus causing an increase in the initiation of lipid oxidation in the treatments that had a low ultimate pH (Juncher et al., 2000).

Drip loss at 2 d postmortem was negatively ($P < 0.05$) correlated to 1 h pH, suggesting that higher drip loss is related to lower 1 h pH ($r = -0.366$). This lower 1 h pH could indicate that these loins had an increased rate of pH decline. The results of this study are in agreement with several other studies. Bowker, Wynveen, Grant, and Gerrard (1999) used electrical stimulation to induce PSE-like conditions in pork carcasses. The loins from carcasses that were electrically stimulated had significantly ($P < 0.001$) lower pH values from

7 min to 56 min postmortem, but displayed no difference ($P > 0.01$) in ultimate pH (Bowker et al., 1999). These loins from electrically stimulated carcasses also showed greater ($P < 0.05$) percent drip loss than the pigs that received no electrical stimulation (Bowker et al., 1999). Lonergan et al. (2001) observed that meat from pigs selected for lean growth efficiency had a significantly lower pH decline than did loins from the control line. The product that had a faster pH decline also had a greater amount of drip loss in the longissimus dorsi (1, 3, and 4 d storage), semitendinosus (2, 3, and 4 d storage), and semimembranosus (1, 2, 3, and 4 d storage). The association between low early postmortem pH and increased drip loss may be due to higher amounts of protein denaturation in muscles that had a faster pH decline. Offer and Knight (1988) found that rapid pH decline while the carcass temperature is still high could cause increased protein denaturation. Extreme denaturation of major contractile proteins such as myosin (Penny, 1969) can cause major texture and water-holding capacity problems in the product. This denaturation of contractile proteins causes the length between actin and myosin to shrink due to the length of the myosin heads decreasing from approximately 19 nm to approxiamately 17 nm (van Laack, 1999). This change in the length of these crossbridges can cause the filament spacing to decrease, resulting in more myofibrillar water to be expelled into the extra-myofibrillar spaces.

Other factors besides the aforementioned microenvironmental conditions within the tissue can affect pork quality such as the extent of proteolysis that occurs in the time postmortem. An increase in protein degradation by μ-calpain can affect both tenderness and water-holding capacity.

μ-Calpain Analysis

The ubiquitous enzyme μ-calpain is a proteolytic enzyme that is activated by the presence of Ca^{2+} (Reverter, Strobl, Fernandez-Catalan, Sorimachi, Suzuki, & Bode, 2002). Calpain molecules undergo the phenomenon of autolysis in the presence of Ca^{2+}. Autolysis results in the cleavage of the N-terminal end of the 80 kDa subunit to yield an active 78 kDa subunit. Once this occurs, 12 more amino acids are removed to yield an active 76 kDa product (Zimmerman & Schlapfer, 1991). Although there is much debate as to the role autolysis plays in the activity of μ-calpain, autolysis has been shown to lower the Ca^{2+} requirement for activity (Cottin, Thomson, Sathe, Szpacenko, & Goll, 2001). This suggests that as the level of autolysis increases, the proteolytic enzyme may be more likely to be active as it is degrading itself.

An increase in the activity of μ-calpain has been shown to increase tenderness in meat products. Koohmarie, Seideman, Schollmeyer, Dutson, and Babiker, (1988) observed that active μ-calpain was able to reproduce the postmortem changes in three different bovine muscles. This was accomplished by extracting μ-calpain from the LD, biceps femoris (BF), and psoas major (PM). The μ-calpain activity from these muscles was compared to the WBS at 1 d and 14 d. The highest aging response (in terms of change in WBS) and highest extractable μ-calpain activity was found in the LD from 1 d to 14 d postmortem, while the lowest aging response and lowest extractable μ-calpain activity was observed in the PM, suggesting that the activity of μ-calpain could explain differences in the aging response of these muscles in beef. A study by Melody et al. (2003) found a similar aging response in the across the same muscles in pork. This study observed that the LD had a greater aging response than the PM between 24 h and 120 h postmortem (Melody et al., 2003). The PM

also had less sarcoplasmic μ-calpain activity at 45 min postmortem than the LD (Melody et al, 2003). Huff-Lonergan et al. (1996) showed that purified μ-calpain was able to degrade certain myofibrillar and myofibril-associated proteins such as titin, nebulin, filamin, desmin, and troponin-T when incubated with purified myofibrils. The increased degradation of these myofibrillar proteins was linked to increased tenderness in these products. An increased oxidative environment can also reduce μ-calpain activity, which may result in a decrease in the rate of μ-calpain autolysis (Guttmann et al., 1997; Guttmann and Johnson, 1998; Rowe et al., 2003).

Differences in level of autolysis of μ-calpain were observed only at 24 h postmortem in the LD. At one h postmortem, all of the samples showed predominately the 80 kDa intact μ-calpain subunit and at 96 h and 7 d postmortem the samples showed only the 76 kDa subunit. However, differences in the degree of autolysis were observed between the samples at 24 h postmortem (Figure 1). The correlations for μ-calpain are described in Table 3.

An increase in μ-calpain autolysis was related to the 1 h pH (Table 3 and Figure 1). More specifically, an increase in autolysis was observed in samples that also had lower pH at 1 h (Figure 1). The presence of the intact 80 kDa subunit of μ-calpain at 24 h postmortem was positively ($P < 0.05$) correlated to pH at 1 h ($r = 0.288$). Also, the presence of the autolyzed 78 kDa subunit of μ-calpain was negatively correlated to 1 h pH ($r = -0.420$). These correlations suggest that products with a lower 1 h pH tended to have an increased degree of μ-calpain autolysis at 24 h. This lower pH may accelerate the activity of μ-calpain (Koohmaraie, 1992; Rowe, Huff-Lonergan, & Lonergan, 2001b; Melody et al., 2003), thus causing a greater likelihood that autolysis is occurring. An increase in autolysis would indicate that μ-calpain is active and is effectively degrading itself, as well as its substrates.

This acceleration in activity may be due to a decrease in the stability of the μ-calpain molecule at a lower pH. This instability resulting in the increased rate of autolysis may ultimately lead to the inactivation of μ-calpain (Geesink & Koohmaraie, 2000).

μ-Calpain autolysis was also related to tenderness of the samples. Samples that had increased autolysis were likely to have lower WBS at 2 d and 21 d (Table 3 and Figure 1). The presence of the intact 80 kDa subunit was positively ($P < 0.05$) correlated to WBS at 2 d ($r = 0.436$) and at 21 ($r = 0.443$) d postmortem. Furthermore, the presence of the autolyzed 76 kDa subunit was negatively ($P < 0.05$) correlated to WBS at 2 d ($r = -0.510$) and at 21 d ($r = -0.498$). These correlations may indicate that samples that had increased μ-calpain autolysis at 24 h also tended to have increased tenderness. This may be a result of increased degradation of myofibril-associated proteins such as desmin (Huff-Lonergan et al., 1996). Degradation of Ca^{2+}-regulating proteins, such as the SERCA and RYR1, may hinder their function and promote an increase in the concentration of Ca^{2+} in the sarcoplasm. This increase in Ca^{2+} could be utilized by μ-calpain for activation of the enzyme.

Several studies have shown that autolysis of μ-calpain parallels its activity. Baki, Tompa, Alexa, Molnar, and Fredrich (1996) measured the degradation of the μ-calpain substrate MAP-2 and used SDS-PAGE to observe the degree of autolysis of human erythrocyte μ-calpain. It was observed that the rate of MAP-2 hydrolysis was a linear function of the amount of the 76 kDa form of μ-calpain and that the 80 kDa form of μ-calpain also showed very minimal activity of the enzyme when measured as degradation of MAP-2 (Baki et al., 1996). This was accomplished by comparing the rate of MAP-2 proteolysis against the percentage of μ-calpain conversion into the 76 kDa form (Baki et al., 1996).

A study by Koohmaraie (1992) used purified μ-calpain from bovine longissimus muscle to compare autolysis with the extent of activation of the enzyme. This study found that a small portion of the 80 kDa subunit was still present after 60 min of incubation with 3.8 mM $CaCl_2$ but was completely degraded at 120 min of incubation. The most intense band observed at 120 min is a 40 kDa band, suggesting that μ-calpain is being autolyzed beyond the 76 kDa in this favorable environment (Koohmaraie, 1992). When compared with the activity of μ-calpain (using casein as a substrate), 79.2% of its initial activity was observed at 120 min (Koohmaraie, 1992). This would suggest that an increase in autolysis would coincide with a reduction in the activity of the enzyme. Furthermore, this reduction in activity could mean that μ-calpain is in fact active and is actually beginning to lose its activity, as it becomes increasingly autolyzed.

Although the results of our study do not prove that autolysis is necessary for activation, it was interesting to note the relationships between the level of autolysis and the biochemical factors (SERCA-1 degradation, ryanodine receptor degradation, desmin degradation) and quality attributes (pH, WBS) observed in this project. More research is needed to fully explain the significance that autolysis has in relation to the activity of μ-calpain postmortem muscle.

Sarcoplasmic Reticulum Ca^{2+}-ATPase Analysis

The sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump is a 110 kDa protein which functions to remove Ca^{2+} from the sarcoplasm and return Ca^{2+} back to the sarcoplasmic reticulum (Berg, Tymoczko, & Stryer, 2002). Three different isoforms of the SERCA pump exist, however SERCA-1 is the isoform found predominately in fast-twitch skeletal muscle

(Brandl, Green, Korczak, & MacLennan, 1987). SERCA functions in the presence of ATP and Ca^{2+} (Berg et al., 2002). Our analysis observed that the 110 kDa intact protein showed little degradation at 1 h and 24 h postmortem. However, degradation products were observed at 96 h and 7 d postmortem in some samples. The correlations for SERCA-1 are shown in Table 4.

SERCA-1 is also susceptible to proteolysis by μ-calpain (Wang et al., 1988a and 1988b; Seidler et al., 1998). The current study showed that an increase in SERCA-1 degradation was related to an increase in the extent of μ-calpain autolysis (Table 4 and Figure 2). SERCA-1 degradation at 7 d was negatively correlated ($P < 0.05$) to the percentage of the unautolyzed 80 kDa form of μ-calpain ($r = -0.272$) at 24 h. Also, SERCA-1 degradation at 7 d was positively ($P < 0.05$) correlated to the percentage of the 76 kDa form of μ-calpain ($r = 0.287$) at 24 h. These correlations suggest that a greater level of μ-calpain autolysis (less 80 kDa and more 76 kDa present) may be related to higher amounts of SERCA-1 degradation. This could mean that as μ-calpain is becoming increasingly autolyzed from the 80 kDa form to the 76 kDa form, it is also causing an increase in the degradation of SERCA-1, possibly due to an increase in sarcoplasmic Ca^{2+} as a result of a loss of SERCA function in early time points postmortem.

It was interesting to note that a higher migrating SERCA-1 degradation product (Deg A, Figure 2) at 7 d postmortem was negatively ($P < 0.05$) correlated to WBS at 2 d and 21 d postmortem ($r = -0.515$ and $r = -0.347$ respectively, Table 4). A similar negative ($P < 0.05$) correlation existed between the lower molecular weight degradation product (Deg B, Figure 2) at 7 d postmortem and WBS at 2 d and 21 d postmortem ($r = -0.497$ and $r = -0.422$ respectively, Table 4). These correlations suggest that increased SERCA-1 degradation may

be related to increased tenderness. Although it is unlikely that the SERCA-1 is still functional at 7 d postmortem due to a lack of ATP, the degradation of SERCA-1 could act as an overall indicator of protein degradation in the product.

Oxidation is detrimental to SERCA activity by causing a reduction in its ability to remove Ca^{2+} from the sarcoplasm to the sarcoplasmic reticulum in living tissue (Klebl et al., 1998). This oxidation could possibly affect the quaternary or tertiary structure of the SERCA in a manner that would not allow ATP binding to occur (Xu et al., 1997; Grover, Samson, Robinson, & Kwan, 2003), thus causing a decrease in SERCA activity. The decrease in the activity of the SERCA pump could create an environment that could be very detrimental to pork quality early postmortem by limiting the amount of Ca^{2+} that could be removed from the sarcoplasm back to the sarcoplasmic reticulum. This would result in a higher concentration of Ca^{2+} in the sarcoplasm, thus promoting muscle contraction and a faster rate of anaerobic metabolism and subsequent early pH decline. Greater lipid oxidation was related to an increase in the presence of SERCA-1 degradation products (Table 4 and Figure 2). This study found positive ($P < 0.05$) correlations between the presence of SERCA-1 degradation 7 d and the concentrations of HNE ($r = 0.322$) and MDA ($r = 0.324$), both measured at 24 h. These correlations may indicate that a shift to a more oxidative environment early postmortem may affect the susceptibility of SERCA-1 to degradation. Also, an increase in oxidation may affect the ATP binding site of the SERCA-1, thus causing the SERCA-1 function to be reduced (Xu et al., 1997). This could then promote an increase in sarcoplasmic Ca^{2+} that could aid in the activation of μ-calpain.

Ryanodine Receptor Analysis

Calcium is released into the sarcoplasm from the lateral cisternae of the sarcoplasmic reticulum by means of the 565 kDa ryanodine receptor (RYR1). The RYR1 is also susceptible to proteolysis by μ-calpain (Gilchrist, Wang, Katz, & Belcastro, 1992; Iino et al., 1992; Shoshan-Barmatz, Weil, Meyer, Varsanyi, & Heilmeyer, 1994). Gilchrist et al. (1992) observed the production of an approximately 410 kDa degradation product and also an approximately 150 kDa degradation product when RYR1 was exposed to both μ-calpain and m-calpain. The study by Iino et al. (1992) reported an increased rate of Ca^{2+} release by the RYR1 when it was degraded by m-calpain. They observed that after the ~410 kDa peptide of the RYR1 was cleaved by m-calpain, the maximum rate of Ca^{2+} release doubled in these samples (Iino et al., 1992). The authors suggested that the N-terminal region, which acts to modulate the opening of the Ca^{2+} channel, might suppress the activity of the RYR1 when the channel is intact (Iino et al., 1992). However, Iino et al. (1992) did not specify if this N-terminal region is cleaved during proteolysis.

Our analysis of the RYR1 revealed differences in degradation of this protein at both 24 h and 96 h (Figure 3). At 24 h postmortem, some samples showed the appearance of a lower molecular weight band (Band B, Figure 3). Differences in the disappearance of a higher molecular weight band (Band A, Figure 3) were noted at 96 h and 7 d.

Oxidation of the RYR1 has been observed to affect the function of the RYR1 in a negative fashion by causing the RYR1 to release large amounts of Ca^{2+} into the sarcoplasm (Donoso et al., 2000; Feng et al., 1998; Zable et al., 1997). If oxidation of the RYR1 occurred early postmortem, this large release of Ca^{2+} could be detrimental to pork quality by resulting in an increase in the rate of postmortem glycolysis and a subsequent increase in the

rate of pH decline. Our study did not find significant ($P > 0.05$) correlations between the level of lipid oxidation and the presence of RYR1 bands A or B. Although the degradation of the ryanodine receptor was not correlated to oxidation, its function may have been affected by oxidation. This could be explained by the negative ($P < 0.05$) correlation between 24 h pH and lipid oxidation. A lower ultimate pH could be due to an increase in muscle metabolism, brought on by a loss of control of the ryanodine receptor. This would result in a large release of Ca^{2+} into the sarcoplasm, promoting muscle metabolism. The loss of control could possibly be due to increased protein oxidation of the ryanodine receptor but not necessarily related to the degradation of the ryanodine receptor.

The relationships between the intensity of band B at 24 h and μ-calpain autolysis can be observed in Table 5. This earlier degradation of RYR1 appears to be related to the extent of μ-calpain autolysis (Figure 4). A greater percentage of the 80 kDa μ-calpain subunit was highly ($P < 0.05$) correlated to band B ($r = -0.654$). The presence of band B was also highly ($P < 0.05$) correlated to the presence of 78 kDa ($r = 0.522$) and 76 kDa ($r = 0.602$) bands. These correlations may indicate that an increase in μ-calpain autolysis may be related to an increase band B, suggesting an increase in degradation of the RYR1. A similar relationship between RYR1 degradation and μ-calpain autolysis was also shown at 96 h (Table 5 and Figure 5). The presence of more of the higher molecular weight band A at 96 h was negatively ($P < 0.05$) correlated to μ-calpain autolysis to the 76 kDa band ($r = -0.297$), suggesting that a less extent of μ-calpain autolysis may be related to less degradation of the RYR1. As previously discussed, the degradation of the RYR1 causes a loss of function and results in an increased release of Ca^{2+} into the sarcoplasm. This higher Ca^{2+} concentration

could be used to activate μ-calpain and could then promote the degradation of the RYR1 and other cytoskeletal proteins that could be associated with tenderness.

It was interesting to note the relationship between WBS and RYR1 degradation (Table 5) at 24 h (Band B, Figure 4) and 96 h (Band A, Figure 5). Samples that showed an increase in band B at 24 h and a decrease in band A at 96 h tended to have lower WBS. This increase in the presence of band B was negatively correlated to WBS at 21 d ($r = -0.306$). The presence of band A at 96 h postmortem was also positively ($P < 0.05$) correlated to WBS at 2 d postmortem ($r = 0.269$). These correlations suggest that more RYR1 degradation may be associated with more tender products. This may be a result of an increase in the activity of μ-calpain, possibly due to a loss of control of the RYR1 and a higher concentration of Ca^{2+} being available for activation of μ-calpain. This would also promote the degradation of other myofibrillar and cytoskeletal proteins such as desmin.

An increase in the presence and intensity of band B at 24 h was related to higher pH at 24 h (Table 5 and Figure 4). Band B at 24 h postmortem was negatively ($P < 0.05$) correlated to pH ($r = 0.303$), suggesting that a higher ultimate pH may be associated with more degradation of RYR1. A possible explanation for this relationship could be that the higher pH may be altering the way calmodulin associates with the RYR1. Calmodulin is a small (17 kDa), ubiquitously expressed Ca^{2+} binding protein that appears to act as a Ca^{2+} sensor in eukaryotic cells (Celio, Pauls, & Schwaller, 1996) and has been found to interact with single RYR1s in lipid bilayers (Smith et al., 1986). Calmodulin binds to a site on the RYR1 that is ~10 nm from the entrance to the transmembrane pore of the RYR1 (Wagenknecht, Grassucci, Frank, Saito, Inui, & Fleischer, 1989). Calmodulin has been shown to activate the ryanodine receptor at submicromolar cytosolic Ca^{2+} concentrations

while inhibiting ryanodine receptor functions at higher Ca^{2+} concentrations (Tripathy, Xu, Mann, & Meissner, 1995). Occupation of the calmodulin-binding site on the RYR1 by calmodulin altered or inhibited calpain degradation that usually occurred at or near the calmodulin-binding site (Wang, Villalobo, & Roufogalis, 1989). A higher pH could result in a modification of the calmodulin-binding site that would interfere with the calmodulin binding, thus promoting the degradation of the RYR1 by μ-calpain. Another possible explanation for this pH-RYR1 relationship could be due to different polymorphisms of the RYR1 gene and also the calpastatin gene. Calpastatin is the natural occurring inhibitor of μ-calpain. Using three different restriction endonuleases, two different polymorphisms were observed for both the RYR1 and for calpastatin (Kocwin-Podiadla, Kuryl, Krzecio, Zybert, & Przybylski, 2003). These polymorphisms resulted in the three different genotypes for both the RYR1 and calpastatin (Kocwin-Podsiadla et al., 2003). A significant statistical ($P < 0.05$) interaction between the RYR1 and calpastatin gene was observed for 45 min pH and drip loss (Kocwin-Podsiadla et al., 2003). These interactions could be a result of the RYR1 genotype or as a result of the activation of the calpain-calpastatin system by the change in Ca^{2+} concentration in the sarcoplasm caused by the RYR1 genotype (Kocwin-Podsiadla et al., 2003). This would suggest that different polymorphisms in both the RYR1 and calpastatin genes could possibly be related to differences in the rate of pH decline and drip loss.

The findings of Iino et al. (1992) along with the observations made in this study suggest that an increase in the degradation of the RYR1 by μ-calpain may promote the release of Ca^{2+} postmortem. This could potentially have both negative and positive ramifications. If this degradation occurred early postmortem, a fully functioning SERCA

pump would need to work to remove the excess Ca^{2+} being released to prevent an increase anaerobic metabolism and thus increase the rate of pH decline. However, this relationship could be a positive factor later postmortem by increasing in the rate of release of Ca^{2+} into the sarcoplasm. This increase in the concentration of Ca^{2+} could overcome Ca^{2+} activation requirements of μ-calpain and possibly m-calpain. μ-Calpain's Ca^{2+} activation requirement is 5-70 μM Ca^{2+}, whereas m-calpain's Ca^{2+} activation requirement is 100-2000 μM (Goll et al., 1992). This may suggest that early postmortem degradation may be a result of μ-calpain activity. However, as the RYR1 is becoming more degraded, more Ca^{2+} is being released into the sarcoplasm. This sarcoplasmic Ca^{2+} concentration could possibly attain the necessary concentration to activate m-calpain as well. More research is needed to better understand the relationships behind the degradation of the RYR1 and calpains and also the biochemical mechanisms behind RYR1 degradation.

Desmin Analysis

Desmin is a 55 kDa intermediate filament protein that is thought to function to maintain the integrity of the muscle cells, thus its degradation may compromise the structure of the muscle cell (Robson, 1995). The degradation product of desmin was not observed until 24 h postmortem (Figure 6). Different degradation patterns observed in this study can be observed in Figure 6. A decrease in the presence of intact desmin coincided with the increased presence of the degradation product

Desmin has been observed to be susceptible to proteolysis by μ-calpain (O'Shea et al., 1979, Huff-Lonergan et al., 1996). Thus, an increase in the activity of μ-calpain would likely promote the degradation of desmin. Table 6 and Figure 6 show a representation of the

relationships between desmin degradation and μ-calpain autolysis. A positive ($P < 0.05$) correlation existed between the percentage of the 80 kDa subunit of μ-calpain and the amount of intact desmin at 96 h and 7 d ($r = 0.400$ and 0.347 respectively). A negative correlation ($P < 0.05$) was observed between the amount of intact desmin at 96 h and 7 d and the presence of the 76 kDa subunit of μ-calpain at 24 h ($r = -0.524$ and –0.494 respectively). The desmin degradation product was negatively ($P < 0.05$) correlated to the percentage of the 80 kDa subunit of μ-calpain at 24 h and 96 h ($r = -0.617$ and –0.313 respectively). Furthermore, the presence of the desmin degradation product was positively ($P < 0.05$) correlated to the presence of the 76 kDa μ-calpain subunit ($r = 0.709$). These correlations indicate that as the extent of μ-calpain autolysis increases, desmin becomes increasingly degraded. This increased degradation of desmin likely means that other cytoskeletal and myofibrillar proteins such as titin, nebulin, filamin, and troponin-T (all are μ-calpain substrates) are likely being degraded as well by μ-calpain. This combined degradation of the ultrastructure of the myofibril could then promote an increase in tenderness of the product, possibly due to the integrity of the myofibril becoming diminished.

The extent of desmin degradation in this study can be related to increased tenderness (Table 6 and Figure 6). Analysis of the desmin degradation product at 24 h showed a negative correlation with WBS at 2 d and at 21 d ($r = -0.348$ and –0.316 respectively). This correlation suggests that an increase in desmin degradation at 24 h was related to lower WBS. Intact desmin at 96 h was positively ($P < 0.05$) correlated to WBS at 2 d ($r = 0.387$) and also to WBS at 21 d ($r = 0.486$). At 7 d, intact desmin was also positively ($P < 0.05$) correlated to WBS at 2 d ($r = 0.290$) and to WBS at 21 d ($r = 0.316$). This correlation suggests that a sample with less intact desmin at later time points postmortem also had lower

WBS. The findings of this study are in agreement with several other studies that have investigated the relationships between desmin degradation and tenderness. Beef samples that had higher WBS values have also been shown to have a higher amount of the 55 kDa intact desmin present (Koohmaraie et al., 1984). In a study done by Huff-Lonergan et al. (1996), beef samples that had significantly lower WBS also had increased desmin degradation and the degradation of desmin occurred more rapidly. Rowe et al. (2001a) showed that an increase in intact desmin is significantly correlated to WBS at 5 d postmortem ($r = 0.295$). Melody et al (2003) found that pork LD samples had significantly less intact desmin at 24 h ($P < 0.05$), 48 h ($P < 0.01$), and 120 h ($P < 0.01$) than the semimembranosus (SM), which may partly account for the differences observed in WBS at 48 h (LD = 3.62 vs. SM = 5.16) and 120 h (LD = 3.41 vs. SM = 3.84). These findings suggest that as the amount of intact desmin increases, the shear force also increases and tenderness decreases.

Conclusions

Properties that distinguish a high quality pork product from a low quality pork product can be complex and influenced by many metabolic factors. This study observed relationships between oxidation and pH, suggesting that oxidation increased with decreased pH. This may be as a result of oxidation of the ryanodine receptor that does not affect its degradation. However, increased oxidation was correlated to increased SERCA-1 degradation in later time points, suggesting that oxidation may be causing a loss of function of the SERCA-1. This loss of function could be promoting its degradation by μ-calpain as a result of an increase in Ca^{2+} concentration in the sarcoplasm overcoming the necessary Ca^{2+}

activation requirement for μ-calpain. This activation of μ-calpain could then promote the degradation of myofibrillar and cytoskeletal proteins such as desmin as well as a further degradation of Ca^{2+}-regulating proteins. Furthermore, this study also showed correlations between μ-calpain autolysis and the degradation of SERCA-1, ryanodine receptor, and desmin. The increased degradation of these proteins was also correlated to increased tenderness in these samples. The data reported in this study are new and novel in that relationships between oxidation, the degradation of Ca^{2+}-regulating proteins, and μ-calpain autolysis in postmortem tissue were observed, which could then be related to an increase in pork quality. These results may aid in directing further research in uncovering some of the biochemical mechanisms that affect the quality of pork being produced. More research is needed to fully understand the postmortem interactions between Ca^{2+}-regulating proteins and μ-calpain and how this interaction may affect pork quality.

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Table 1
Means, minimums, maximums, and standard deviations of variables used in Pearson correlation analysis of the longissimus dorsi (LD).

Variable	N	Mean	Min	Max	Standard Deviation
pH 1 h	50	6.28	5.47	6.92	0.26
pH 24 h	54	5.49	5.36	5.89	0.09
% Drip Loss 2 d	54	0.63	0.00	1.55	0.31
WBS 2 d (Kg)	53	4.16	2.61	6.30	0.73
WBS 21 d (Kg)	52	3.30	2.43	5.70	0.59
Total Glycosyl Units 1 h (μM)	51	26.74	7.08	46.14	9.84
Total Glycosyl Units 24 h (μM)	52	8.11	-5.86	33.29	6.52
4-HNE 24 h (mg/kg)	54	0.16	0.10	0.23	0.03
MDA 24 h (mg/kg)	54	0.22	0.13	0.31	0.04
SERCA-1 Deg A 7 d	53	63.88	0.00	171.35	38.60
SERCA-1 Deg B 7 d	53	47.75	0.00	145.50	33.54
Intact Desmin 1 h	52	97.19	49.91	160.24	25.76
Intact Desmin 24 h	52	110.75	44.79	205.69	46.52
Intact Desmin 96 h	54	56.49	9.09	122.05	30.40
Intact Desmin 7 d	54	52.80	9.12	115.41	25.20
Desmin Deg 1 h	52	0.00	0.00	0.00	0.00
Desmin Deg 24 h	52	48.99	0.00	375.95	71.72
Desmin Deg 96 h	54	232.53	0.00	520.68	102.77
Desmin Deg 7 d	54	259.95	100.00	513.02	98.51
RYR Band B 24 h	51	84.25	0.00	373.48	79.32
RYR Band A 96 h	54	527.46	0.00	2198	457.88
μ-Calpain 80 kDa 24 h	52	57.66	0.00	100.00	21.37
μ-Calpain 78 kDa 24 h	52	28.41	0.00	71.70	12.64
μ-Calpain 76 kDa 24 h	52	13.94	0.00	56.50	12.20

Table 2
Pearson correlations between the LD quality attributes for 1 h pH, 24 h pH, drip loss at 2 days postmortem, Warner-Bratzler Shear at 2 days and 21 days postmortem, and mg/Kg 4-HNE and MDA at 24 h postmortem.

	pH- 1h	**pH-24h**	**Drip Loss – 2 d**	**WBS-2d**	**WBS-21 d**	**HNE-24h**	**MDA-24h**
pH- 1h	x	0.020	**-0.366***	0.150	0.139	0.126	-0.005
pH-24h	0.020	x	-0.244	0.160	-0.090	**-0.349***	**-0.367***
Drip Loss – 2 d	**-0.366***	-0.244	x	-0.138	0.043	0.109	0.120
WBS-2d	0.150	0.160	-0.138	x	**0.367***	**-0.291***	-0.261
WBS-21 d	0.139	-0.090	0.043	**0.367***	x	0.114	0.127
HNE-24h	0.013	**-0.349***	0.109	**-0.291***	0.114	x	**0.991***
MDA-24h	-0.005	**-0.367***	0.120	-0.261	0.127	**0.991***	x

*Significant correlation between X and Y at the $P < 0.05$ level

Table 3
Pearson correlations between LD 1 h pH, 24 h pH, drip loss at 2 days postmortem, WBS at 2 days postmortem, WBS at 21 days postmortem and mg/Kg 4-HNE and MDA at 24 h postmortem (X) and LD μ-calpain autolysis (Y).

	pH-1h	**pH-24 h**	**Drip Loss -2 d**	**WBS- 2d**	**WBS- 21d**	**HNE-24h**	**MDA-24h**
μ-Calpain 80 kDa –24h	**0.288***	-0.235	-0.175	**0.436***	**0.443***	0.068	0.076
μ-Calpain 78 kDa –24h	**-0.420***	0.247	**0.299***	-0.245	-0.269	-0.037	-0.026
μ-Calpain 76 kDa –24h	-0.063	0.155	-0.004	**-0.510***	**-0.498***	-0.080	-0.105

*Significant correlation between X and Y at the $P < 0.05$ level.

Table 4
Pearson correlations between LD 1 h pH, 24 h pH, drip loss at 2 days postmortem, WBS at 2 days postmortem, WBS at 21 days postmortem, mg/Kg 4-HNE and MDA at 24 h postmortem, and μ-calpain autolysis at 24 h (X) and LD SERCA-1 degradation at 7d (Y).

*Significant correlation between X and Y at the $P < 0.05$ level

	pH-1h	**pH-24h**	**Drip Loss –2d**	**WBS-2d**	**WBS-21d**	**HNE - 24h**	**MDA-24h**	**μ-Calpain 80 kDa –24 h**	**μ-Calpain 78 kDa –24 h**	**μ-Calpain 76 kDa –24 h**
SERCA-1 Deg A- 7 d	0.043	-0.145	-0.046	**-0.515***	**-0.347***	0.217	0.199	-0.193	0.106	0.230
SERCA-1 Deg B- 7 d	0.010	-0.216	0.072	**-0.497***	**-0.422***	**0.322***	**0.324***	**-0.272***	0.189	**0.287***

Table 5
Pearson correlations between LD pH at 1 h, pH at 24 h, drip loss at 2 days postmortem, WBS at 2 days postmortem, WBS at 21 days postmortem, mg/Kg 4-HNE and MDA at 24 h postmortem, and μ-calpain autolysis (X) and LD ryanodine receptor degradation A at 24 h and degradation B at 96 h (Y).

	pH-1h	**pH-24h**	**Drip Loss–2d**	**WBS-2d**	**WBS-21d**	**HNE – 24h**	**MDA - 24h**	**μ-Calpain 80 kDa –24 h**	**μ-Calpain 78 kDa –24 h**	**μ-Calpain 76 kDa –24 h**
RYR Band B- 24h	-0.069	**0.303***	0.152	-0.152	**-0.306***	-0.104	-0.117	**-0.654***	**0.522***	**0.602***
RYR Band A- 96h	-0.024	-0.032	0.088	**0.269***	0.128	0.165	0.190	0.187	-0.040	**-0.297***

*Significant correlation between X and Y at the $P < 0.05$ level

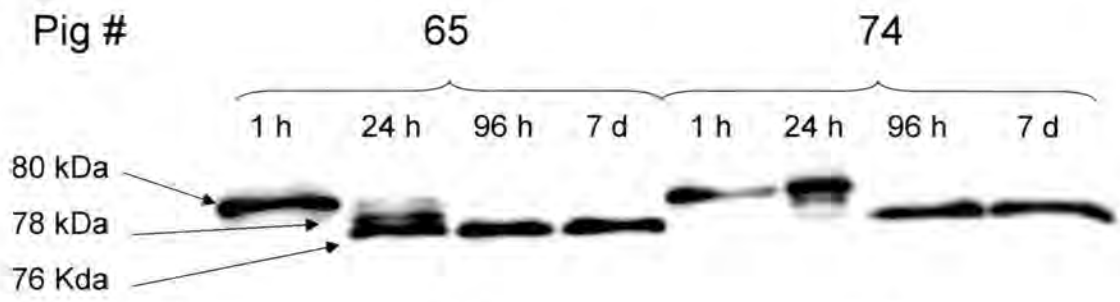
Table 6
Pearson correlations between LD 1 h pH, 24 h pH, drip loss at 2 days postmortem, WBS at 2 days postmortem, WBS at 21 days postmortem, mg/Kg 4-HNE and MDA at 24 h postmortem and μ-calpain autolysis at 24 h (X) and LD desmin degradation (Y).

	pH- 1h	**pH-24h**	**Drip Loss – 2 d**	**WBS-2d**	**WBS-21 d**	**HNE-24h**	**MDA-24h**	**μ-Calpain 80 kDa –24 h**	**μ-Calpain 78 kDa –24 h**	**μ-Calpain 76 kDa –24 h**
Intact Desmin– 1 h	**0.311***	-0.250	-0.010	-0.212	-0.031	-0.073	-0.073	0.053	-0.152	0.065
Intact Desmin-24 h	0.184	-0.055	-0.018	-0.075	0.234	0.041	0.068	-0.005	0.069	-0.061
Intact Desmin-96 h	0.050	-0.117	0.036	**0.387***	**0.486***	0.042	0.049	**0.400***	-0.172	**-0.524***
Intact Desmin-7 d	-0.031	-0.114	-0.069	**0.290***	**0.316***	0.086	0.099	**0.347***	-0.110	**-0.494***
Desmin Deg 24 h	-0.117	0.083	0.143	**-0.348***	**-0.316***	-0.037	-0.041	**-0.617***	**0.353***	**0.709***
Desmin Deg 96 h	**-0.286***	-0.115	0.252	-0.114	-0.050	-0.121	-0.119	**-0.313***	**0.282***	0.255
Desmin Deg 7 d	-0.118	0.091	0.005	-0.199	-0.232	0.012	0.039	-0.134	0.132	0.097

*Significant correlation between X and Y at the $P < 0.05$ level

Figure 1. Western blot of LD samples representing two pigs with different μ-calpain autolysis patterns at 24 h postmortem in the LD. Each lane of the 9% SDS-PAGE gels was loaded with 100 μg of protein for each sample. Listed below each pig is a representation of significant correlations between μ-calpain autolysis at 24 h and 1 h pH, WBS values at 2 d postmortem, and WBS at 21 d postmortem.

Figure 1. Western blot of LD μ-calpain at 1 h, 24 h, 96 h, and 7 d. A representation of significant correlations with LD μ-calpain autolysis at 24 h is listed below each pig.



pH 1 h	6.37	6.49
WBS 2 d (Kg)	2.95	5.55
WBS 21 d (Kg)	2.48	3.98

Figure 2. Western blot of LD samples representing two pigs with different SERCA-1 degradation patterns at 96 h and 7 d postmortem in the LD. Each lane of the 8% SDS-PAGE gels was loaded with 60 μg of protein for each sample (Figure 2a). Listed below each pig is a representation of significant correlations between SERCA-1 degradation at 7 d and lipid oxidation (HNE and MDA), μ-calpain autolysis at 24 h, WBS at 2 d postmortem, and WBS at 21 d postmortem (Figure 2b).

Figure 2a. Western blot of LD SERCA-1 at 1 h, 24 h, 96 h, and 7 d.

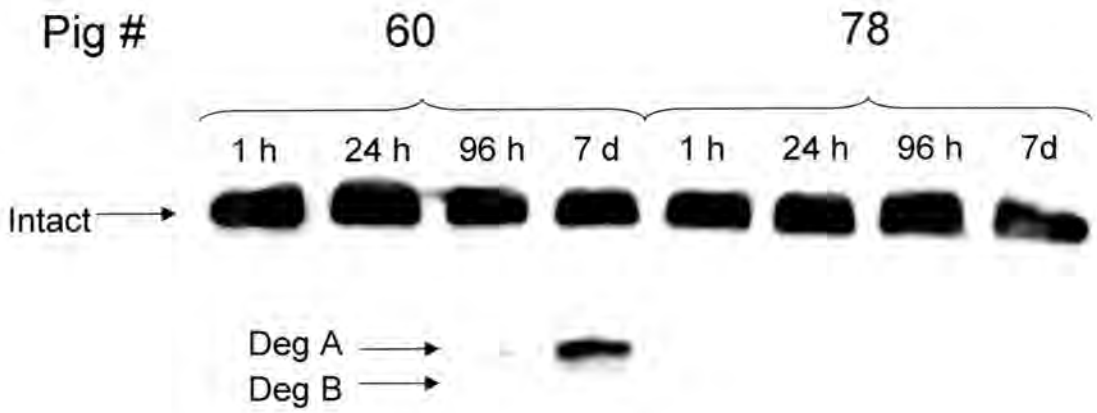


Figure 2b. Representation of significant correlations with LD SERCA-1 degradation including a western blot of LD μ-calpain at 24 h, HNE and MDA (lipid oxidation) at 24 h, and WBS at 2 d and 21 d.

HNE 24 h (mg/Kg)	0.19	0.12
MDA 24 h (mg/Kg)	0.26	0.17
WBS 2 d (Kg)	2.61	5.03
WBS 21 d (Kg)	2.43	3.42

Figure 3. Western blot of LD ryanodine receptor at 1 h, 24 h, 96 h, and 7 d postmortem. Each lane of the 6% SDS-PAGE gels was loaded with 160 μg of protein for each sample. This is a representation of samples from two pigs that display different degradation patterns over all of the time points measured. The last lane (C) is 160 μg of protein of an ovine at death control sample.

Figure 3. Western blot of LD ryanodine receptor at 1 h, 24 h, 96 h, and 7 d.

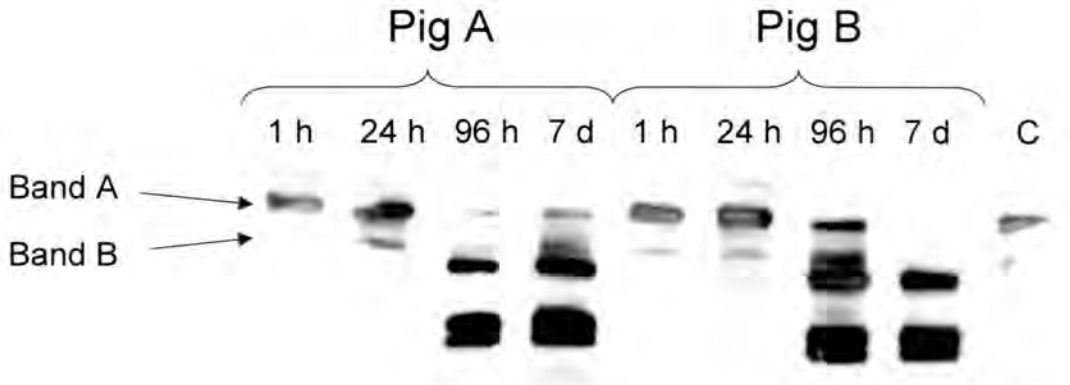


Figure 4. Western blot of LD samples representing 4 pigs with different densitometric intensities of ryanodine receptor (Band B) at 24 h postmortem in the LD. Each lane of the 6% SDS-PAGE gels was loaded with 160 μg of protein for each sample (Figure 4a). The first lane (C) is 160 μg of protein of a porcine control sample at 24 h. Listed below each pig is a representation of significant correlations between ryanodine receptor Band B at 24 h and μ-calpain autolysis at 24 h, 24 h pH, and WBS at 21 d postmortem (Figure 4b).

Figure 4a. Western blot of LD ryanodine receptor at 24 h.

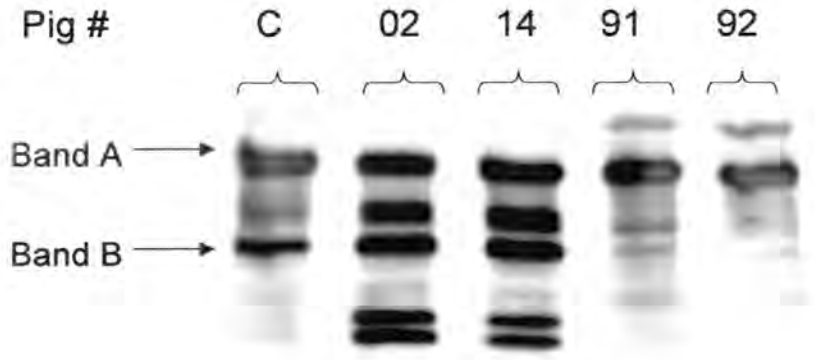


Figure 4b. Representation of significant correlations with LD ryanodine receptor Band B at 24 h including a western blot of LD μ-calpain at 24 h, pH at 24 h, and WBS at 21 d.

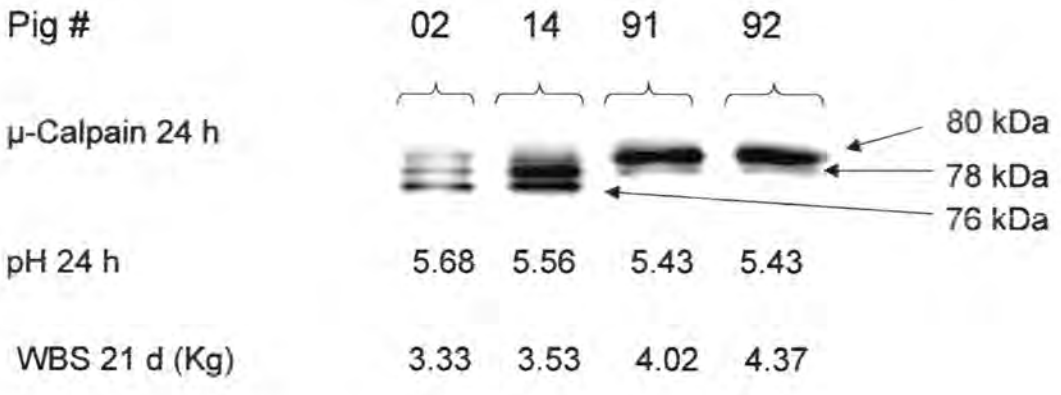


Figure 5. Western blot of LD samples representing 4 pigs with different densitometric intensities of ryanodine receptor (Band A) at 96 h postmortem in the LD. Each lane of the 6% SDS-PAGE gels was loaded with 160 μg of protein for each sample (Figure 5a). The first lane (C) is 160 μg of protein of a porcine control sample at 24 h. Listed below each pig is a representation of significant correlations between ryanodine receptor Band A at 96 h and μ-calpain autolysis at 24 h and WBS at 2 d postmortem (Figure 5b).

Figure 5a. Western blot of LD ryanodine receptor at 96 h.

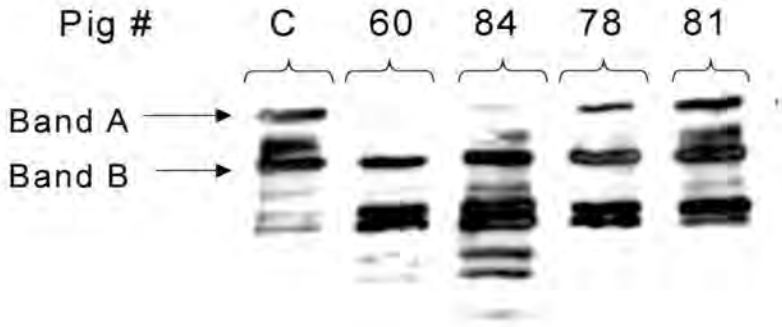


Figure 5b. Representation of significant correlations with LD ryanodine receptor Band A at 96 h including a western blot of LD μ-calpain at 24 h and WBS at 2 d.

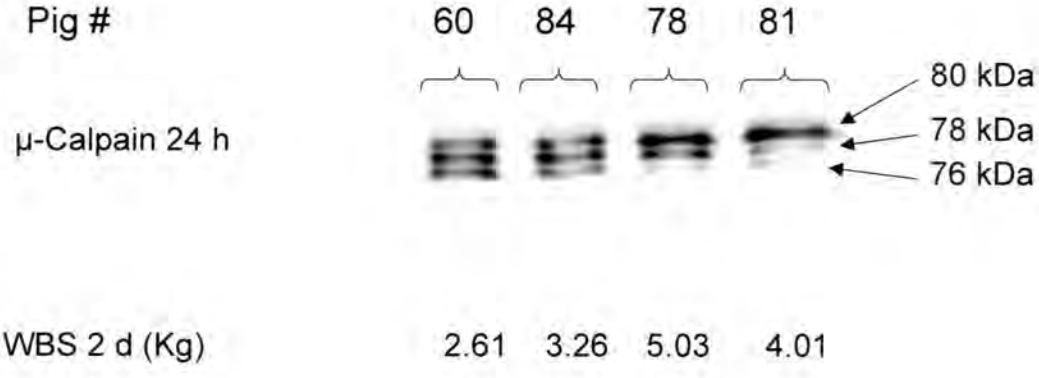


Figure 6. Western blot of LD desmin from 2 pigs at 1 h, 24 h, 96 h, and 7d. This western blot represents samples from 2 pigs with different desmin degradation patterns across time points measured. Each lane of the 10% SDS-PAGE gels was loaded with 30 μg of protein for each sample (Figure 6a). Listed below each pig is a representation of significant correlations between desmin degradation and μ-calpain autolysis at 24 h, WBS at 2 d postmortem, and WBS at 21 d postmortem are listed below each pig (Figure 6b).

Figure 6a. Western blot of LD desmin at 1 h, 24 h, 96 h, and 7 d.

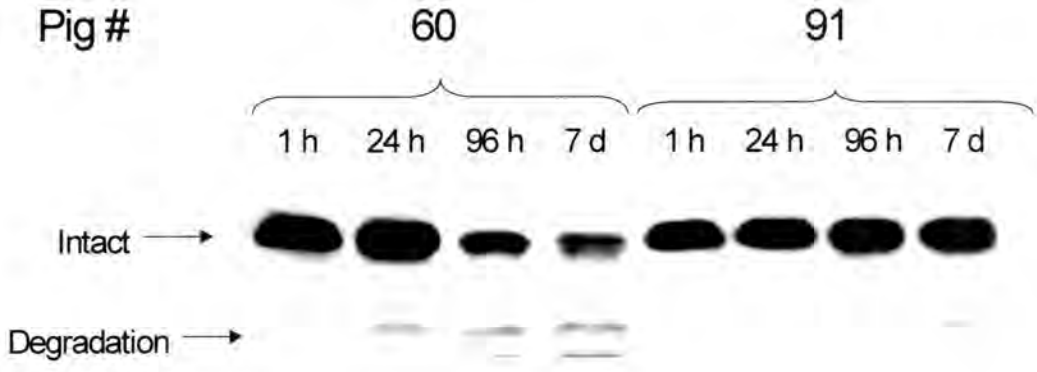
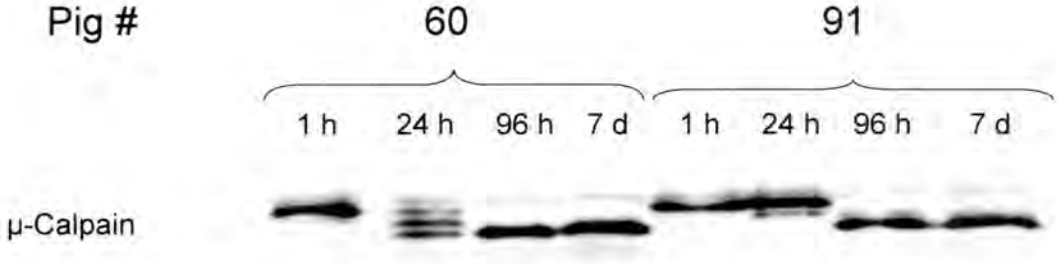


Figure 6b. Representation of significant correlations with LD desmin degradation including a western blot of LD μ-calpain across time and WBS at 2 d and 21 d.



WBS 2 d (Kg)	2.61	4.76
WBS 21 d (Kg)	2.43	4.02

GENERAL SUMMARY

Relationships between pork quality attributes such as oxidation, pH decline, Warner-Bratzler shear force and drip loss were made in this study. Samples that had a lower 1 h pH also tended to display higher drip loss. This could have been a result of increased protein denaturation that may have occurred as a consequence of a fast pH decline while the carcass temperature was still high. Also, increased oxidation was related to decreased pH at 24 h postmortem. This could be attributed to a higher rate of metabolism that caused an increase in the formation of reactive oxygen species while trying to produce ATP. Increased oxidation was also correlated to a decrease in Warner-Bratzler shear force. This may have occurred as a result of oxidation of Ca^{2+}-regulating proteins that resulted in an increase in sarcoplasmic Ca^{2+} and promoting the activity of μ-calpain.

Relationships between μ-calpain autolysis and the degradation of Ca^{2+}-regulating proteins were also made. The ryanodine receptor functions as a Ca^{2+} release channel that releases Ca^{2+} from the lateral cisternae of the sarcoplasmic reticulum into the sarcoplasm. This Ca^{2+} release occurs upon a stimulus to begin muscle contraction. The function of the sarcoplasmic reticulum Ca2+-ATPase-1 (SERCA-1) is to remove Ca^{2+} from the sarcoplasm to the sarcoplasmic reticulum in the presence of ATP to initiate relaxation of the muscle. An increase in the extent of μ-calpain autolysis was correlated to increased SERCA-1 degradation at 7d postmortem. Furthermore, increased ryanodine receptor degradation was correlated to further autolysis of μ-calpain. It could then be hypothesized that the increase in μ-calpain autolysis could have been aided by an increase in Ca^{2+} released into the sarcoplasm

by the ryanodine receptor coupled with a decrease in the activity of the SERCA-1. This loss of inhibition of these two Ca^{2+} regulating proteins would result in an increase in sarcoplasmic Ca^{2+} that could be high enough to activate μ-calpain. This decrease in function of the SERCA-1 could have been a result of oxidation of the SERCA-1. This study observed that increased lipid oxidation was correlated to the degradation of SERCA-1 at 7 d. Oxidation may affect SERCA function by preventing phosphorylation of the enzyme, resulting in a decrease in its activity and promoting the activity of μ-calpain. The increase in the activity of μ-calpain would likely promote proteolysis of myofibrillar and myofibril-associated proteins as well as membranous Ca^{2+}-regulating proteins. Increased proteolysis could result in increased tenderness of the product.

Relationships between μ-calpain autolysis and the degradation of the cytoskeletal protein desmin and Ca^{2+}-regulating proteins can be made from this study. The extent of LD μ-calpain autolysis at 24 h was significantly correlated to pH, suggesting that an increase in autolysis occurred in samples that had low pH at 1 h. This increase in autolysis can be related to an increase in the activity of the proteolytic enzyme.

Decreased Warner-Bratzler shear force at both 2 d and 21 d postmortem was also related to increased μ-calpain autolysis. This lower shear force may result as an increase in proteolysis of myofibrillar and cytoskeletal proteins such as titin, nebulin, filamin, desmin, and troponin-T. The intermediate filament protein desmin functions to maintain the structural integrity of the muscle cells, thus increased degradation may promote increased tenderness. This study observed that an increase in the degradation of desmin was correlated to both an increase in the extent of μ-calpain autolysis and also lower Warner-Bratzler shear force at 2 d and 21 d postmortem. Increased tenderness was observed in samples that

displayed increased SERCA-1 degradation at 7 d postmortem and ryanodine receptor degradation at 24 h and 96 h postmortem. This increased tenderness also coincided with an increase in μ-calpain autolysis in samples that had high SERCA-1 degradation and ryanodine receptor degradation. In conclusion, increased μ-calpain autolysis and increased desmin degradation may be indicators of increased tenderness. Furthermore, as μ-calpain autolysis increases, SERCA-1 and ryanodine receptor degradation may be indicators of increased tenderness.

These observations suggest that early postmortem biochemical factors such as oxidation and pH decline may affect pork quality attributes by altering Ca^{2+} regulation in the muscle, thus promoting the activity of μ-calpain and the subsequent degradation of desmin and Ca^{2+}-regulating proteins. This increase in proteolysis could be used as a predictor of tenderness.

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